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(54) **Recombinant birnavirus vaccine**

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Vaccin recombinant du birnavirus

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The file contains technical information submitted after the application was filed and not included in this specification

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## Description

[0001] The present invention is concerned with a birnavirus mutant, a vaccine comprising this mutant, a method for determining birnavirus infection in an animal, as well as with a test kit for carrying out this method.

[0002] Infectious bursal disease virus (IBDV) and Infectious pancreatic necrosis virus (IPNV) are members of the Birnaviridae family. Viruses in this family have a very similar genomic organisation and a similar replication cycle. The genomes of these viruses consist of 2 segments (A and B) of double-stranded (ds) RNA. The larger segment A encodes a polyprotein which is cleaved by autoproteolysis to form mature viral proteins VP2, VP3 and VP4 (Hudson, P.J. et al, Nucleic Acids Res., 14, 5001-50012, 1986; Dobos P., Annual review of fish diseases 5, 25-54, 1995). VP2 and VP3 are the major structural proteins of the virion. VP2 is the major host-protective immunogen of birnaviruses, and contains the antigenic regions responsible for the induction of neutralising antibodies. The VP4 protein appears to be a virus-coded protease that is involved in the processing of a precursor polyprotein of the VP2, VP3 and VP4 proteins. The larger segment A possesses also a second open reading frame (ORF), preceding and partially overlapping the polyprotein gene. This second open reading frame encodes a protein VP5 of unknown function that is present in IBDV infected cells (Mundt, E. et al., J. Gen. Virol., 76, 437-443, 1995).

[0003] The smaller segment B encodes VP1, a 90 kDa multifunctional protein with polymerase and capping enzyme activities (Spies, U. et al., Virus Res., 8, 127-140, 1987 and Spies, U. et al., J. Gen. Virol., 71, 977-981, 1990; Duncan R. et al., Virology 181, 541-552, 1991).

[0004] For IBDV, two serotypes exist, serotype 1 and 2. The 2 serotypes may be differentiated by virus neutralisation (VN) tests. Furthermore, subtypes of serotype 1 have been isolated. These so-called "variant" viruses of serotype 1 can be identified by cross-neutralisation tests (Diseases of Poultry, 9th edition, 1991, Wolfe Publishing Ltd, ISBN 0 7234 1706 7, Chapter 28, P.D. Lukert and Y.M. Saif, 648-663), a panel of monoclonal antibodies (Snyder, D.B. et al., Arch. Virol., 127, 89-101, 1992.) or RT-PCR (Jackwood, D.J., Proceedings of the International symposium on infectious bursal disease and chicken infectious anaemia, Rauischholzhausen, Germany, 155-161, 1994). Some of these subtypes of serotype 1 of IBDV have been described in literature for example: Classical, Variant-E, GLS, RS593 and DS326 strains (Van Loon, et al. Proceedings of the International symposium on infectious bursal disease and chicken infectious anaemia, Rauischholzhausen, Germany, 179-187, 1994).

[0005] Infectious Bursal disease (IBD), also called Gumboro disease, is an acute, highly-contagious viral infection in chickens that has lymphoid tissue as its primary target with a selective tropism for cells of the bursa of Fabricius. The morbidity rate in susceptible flocks is high, with rapid weight loss and moderate mortality rates. Chicks that recover from the disease may have immune deficiencies because of the destruction of the bursa of Fabricius which is essential to the defence mechanism of the chicken. The IBD-virus causes severe immunosuppression in chickens younger than 3 weeks of age and induces bursal lesions in chicks up to 3 months old.

[0006] For many years the disease could be prevented by inducing high levels of antibodies in breeder flocks by the application of an inactivated vaccine, to chickens that had been primed with attenuated live IBDV vaccine. This has kept economic losses caused by IBD to a minimum. Maternal antibodies in chickens derived from vaccinated breeders prevents early infection with IBDV and diminishes problems associated with immunosuppression. In addition, attenuated live vaccines have also been used successfully in commercial chicken flocks after maternal antibodies had declined.

[0007] Recently, very virulent strains of IBDV have caused outbreaks of disease with high mortality in Europe. The current vaccination programs failed to protect chicks sufficiently. Vaccination failures were mainly due to the inability of live vaccines to infect the birds before challenge with virulent field virus.

[0008] Eradication of the disease by other preventative measures than vaccination has not been feasible, because the virus is widely spread and because with currently administered live attenuated or inactivated IBDV vaccines it is not possible to determine whether a specific animal is infected with an IBDV field virus or whether the animal was vaccinated with an IBDV vaccine. In order to be able to start an eradication control programme for IBDV it is highly desirable that the possibility exists to discriminate between animals vaccinated with an IBDV vaccine and those infected with a field virus so as to be able to take appropriate measures, i.e. remove infected flocks, to reduce spreading of the virulent field virus. The introduction of, for example, a serologically identifiable marker can be achieved by introducing a mutation in genes encoding non-essential (glyco)proteins of the IBDV which still give rise to the production of antibodies in an infected host animal. A marker vaccine for Aujeszky's disease and companion diagnostic tests have proven their practical value in the control of this disease. Whereas such control programs for other viral infectious diseases in animals are under development, until the present invention a vaccine based on an IBDV vaccine strain which would fit in IBDV control programs has not been described yet.

[0009] Mundt and Köllner (Annual Meeting of the German Federal Research Institute for virus diseases in animals, March 1997) disclose the construction of an IBDV mutant that fails to express a VP5 protein as a result of a genetically engineered mutation in the start codon of the VP5 gene. This mutation comprises the substitution of one nucleotide in the start codon of the VP5 gene.

**[0010]** The present invention provides a birnavirus mutant as defined in the claims which is not able to produce a native VP5 protein as a result of a mutation in the VP5 gene of the birnavirus genome.

**[0011]** Preferably, the birnavirus mutant is an IBDV mutant or an IPNV mutant, the IBDV mutant being most preferred, in particular an IBDV mutant derived from a serotype 1 IBD virus is provided by the present invention.

**[0012]** It is demonstrated that an IBDV mutant that is not able to produce a VP5 protein is still able to infect poultry and to replicate in the infected host animals *in vivo*, i.e. evidence is provided that the gene encoding the VP5 protein is a non-essential gene. Example 3 and 4 show that VP5<sup>-</sup> IBDV can be re-isolated from organs of animals infected with the IBDV mutant and that the IBDV mutant induces a protective immune response in the infected animals.

**[0013]** Moreover, it has been established herein that part of the normal anti-IBDV immune response in poultry is directed to the VP5 region. This is rather surprising as the VP5 protein is considered to represent a non-structural viral protein (Mundt et al., J. Gen. Virol. 76, 437-443, 1995) and the immune response in an animal against a viral pathogen is usually elicited against the structural (glyco)proteins of the virus. These findings make the IBDV mutant and other birnavirus mutants according to the present invention a suitable vaccine candidate for a marker vaccine. Such a marker vaccine provides the possibility to determine whether animals are infected with a wild-type birnavirus, e.g. IBDV, or with a vaccine virus.

**[0014]** Additionally, it has been found that the VP5 protein is involved in the expression of virulence of the birnaviruses, in particular of IBDV, and that the inability of the virus mutants to produce the native VP5 protein leads to an attenuation of the virus.

**[0015]** With the term "which is not able to produce a native VP5 protein" is meant that the birnavirus mutant produces a polypeptide that can be distinguished by serological tests from the native VP5 protein, or does not produce a VP5 protein at all. For example, in the former case, the birnavirus mutant produces only a fragment of the native birnavirus VP5 protein which lacks one or more immunogenic epitopes.

**[0016]** Preferably, the birnavirus mutant according to the invention produces no VP5 protein upon infection of a host cell.

**[0017]** As described above, the genomic organisation of the birnaviruses is well established: the IBDV and IPNV genome comprises a large segment A and a smaller segment B. The segment A of IBDV comprises a large open reading frame (ORF) encoding a polyprotein of about 110 kDa (VP2-VP4-VP3). The gene encoding the VP5 protein is identified in the prior art, and defined herein, as the small ORF on segment A of the birnavirus genome which precedes and partially overlaps the polyprotein encoding ORF (Bayliss et al., J. Gen. Virol. 71, 1303-1312, 1990; Spies et al., J. Gen. Virol. 71, 977-981, 1990; Havarstein L.S. et al., J. Gen. Virology 71, 299-308, 1990; Dobos et al., 1995, *supra*; Figures 1-3 herein and SEQ ID No.'s 1-7). The mutation introduced in the VP5 gene is such that it does not prevent the expression of the polyprotein.

**[0018]** SEQ ID No. 1 comprises the full length cDNA nucleotide sequence of segment B of IBDV strain P2, as well as the amino acid sequence of the VP1 protein encoded by segment B (see also SEQ ID. No. 2). SEQ ID No. 3 and 5 depict the full length cDNA sequence of segment A of IBDV strain D78 and the coding region of the VP5 protein and the polyprotein, respectively. SEQ ID 3 and 4 also show the amino acid sequence of the D78 VP5 protein. SEQ ID No. 5 and 6 show the amino acid sequence of the polyprotein VP2-VP4-VP3 of D78. SEQ ID No. 7 shows the 5'-end of segment A of strain D78, including the mutations introduced in the VP5 coding region. SEQ ID No. 8 shows the nucleotide sequence of segment B of strain D78 and the amino acid sequence of the D78 VP1 protein. The genomic organisation of both segments is also shown in Figure 1.

**[0019]** The ORF coding for VP5 is conserved in all hitherto published segment A sequences. The IBDV ORF encodes 145 amino acids resulting in a calculated molecular mass of 16.5 kDa. The nucleotide sequence of the ORF encoding the VP5 protein of IBDV strain D78 used herein is shown in SEQ ID No. 3 and 4. Natural variations may exist between individual IBDV isolates. These natural variations result from small differences in the genomes of these viruses. The nucleotide sequence of the segment A, including the nucleotide sequence of the VP5 gene for many IBDV isolates have been described in the prior art (Vakharia et al., Avian Diseases 36, 736-742, 1992; Bayliss et al., J. Gen. Virol. 71, 1303-1314, 1990; Hudson et al., Nuc. Acid Res. 14, 5001-5012, 1986; Schnitzler et al., J. Gen. Virol. 47, 1563-1571, 1993; Kibenge et al., J. Gen. Virol. 71, 569-577, 1990 and Virology 184, 437-440, 1991; Mundt et al., Virology 209, 10-18, 1995; Lana et al., Virus Genes 6, 247-259, 1992; Vakharia et al., Virus Res. 31, 265-273, 1994; Brown et al., Virus Res. 40, 1-15, 1996). The amino acid sequence of the VP5 protein from serotype I IBDV strains display a homology of at least 95% with the VP5 amino acid sequence shown in SEQ ID No. 3 and 4, whereas the homology between serotype II VP5 sequence and the amino acid sequence shown in SEQ ID No. 3 and 4 is at least 75%. Therefore, a preferred IBDV mutant according to the present invention is an IBDV mutant wherein the mutation is introduced in the VP5 gene having a homology of at least 75%, in particular at least 95% on the amino acid sequence level with the VP5 amino acid sequence shown herein.

**[0020]** Preferably an IBDV mutant according to the present invention is derived from any of the classical or variant (e.g. variant E or GLS) IBDV vaccine strains, such as those currently used in the field. Such suitable IBDV strains include the IBDV vaccine strains present in the commercially available vaccines: D78, PBG 98, LZ 228E, 89-03 (Intervet

International B.V.), Bursine 2 (Fort Dodge Animal Health) and S 706 (Rhône Mérieux).

**[0021]** A particular preferred IBDV mutant according to the invention is derived from the D78 strain comprising a VP5 gene encoding a protein having the amino acid sequence shown in SEQ ID No. 3 and 4.

**[0022]** Alternatively, the parent birnavirus strain for the virus mutant according to the invention is a virulent birnavirus field strain. It is found herein that the VP5 protein is a factor associated with virulence, and that the absence of the native VP5 protein in a birnavirus results in an attenuated form of the virus.

**[0023]** Preferably the invention provides a birnavirus mutant which is not able to produce a native VP5 protein as a result of a mutation in the part of the VP5 gene which does not overlap with the large ORF encoding the polyprotein.

**[0024]** In particular, the birnavirus mutant according to the invention comprises a mutation in the 5'-end of the VP5 gene spanning nucleotides 1-30, preferably 1-20, more preferably 1-10. Most preferred is an birnavirus mutant having a mutation in nucleotides 1-3 of the VP5 gene.

**[0025]** A mutation is understood to be a change of the genetic information in the VP5 gene with respect to the genetic information present in this region of the genome of naturally occurring birnavirus producing native VP5 protein. The mutation is, for example, a nucleic acid substitution, deletion, insertion or inversion, or a combination thereof.

**[0026]** In a preferred embodiment of the present invention a birnavirus mutant is provided wherein the mutation is a substitution of one or more nucleotides. In particular, a nucleic acid substitution is introduced in the start codon, as a result of which the new codon encodes an amino acid different from methionine or represents a stop codon, preferably the nucleic acid substitution comprises at least two of the nucleotides of the start codon.

**[0027]** A further birnavirus mutant according to the invention comprises a substitution of one or more nucleotides in a codon(s) different from the start codon resulting in one or more stop codons, preferably in the 5'-end of the VP5 gene as defined above, if desired in addition to a substitution in the start codon as described above. Preferably, the birnavirus mutant comprises a stop codon in this region of the VP5 gene in each of the three reading frames.

**[0028]** Such a preferred birnavirus mutant may be an IBDV mutant having a mutation in the start codon, the fourth and the sixth codon of the VP5 gene, preferably resulting in the mutated codons shown in SEQ ID No. 7 and Figure 3.

**[0029]** Alternatively, a birnavirus mutant is provided wherein the mutation is a deletion. In particular, the deletion comprises less than 20, less than 10 or less than 5 nucleotides. Preferably, the deletion comprises a total number of nucleotides not dividable by three, resulting in a shift of the reading frame.

**[0030]** Preferably the deletion comprises one or more nucleotides of the start codon of the VP5 gene.

**[0031]** In an alternative embodiment of the present invention a birnavirus mutant is provided wherein the mutation comprises the insertion of a heterologous nucleic acid sequence in the birnavirus genome. A heterologous nucleic acid sequence is a nucleic acid sequence normally not present at the specific insertion site of the particular virus species.

**[0032]** The heterologous nucleic sequence to be incorporated into the birnavirus genome is a nucleic acid fragment which either encodes a polypeptide or is a non-coding sequence. The nucleic acid fragment can be derived from any source, e.g. viral, eukaryotic, prokaryotic or synthetic, including oligonucleotides suitable for the interruption of the expression of the VP5 gene.

**[0033]** A suitable oligonucleotide for the interruption of the VP5 expression may comprise three translational stop codons in each of the possible reading frames in both directions, in addition to one or more appropriate restriction enzyme cleavage sites useful for the insertion of a second heterologous nucleic acid sequence. The length and nucleotide sequence of such a non-coding heterologous nucleic acid sequence is not critical, but preferably varies between 8-50 nucleotides.

**[0034]** In a further embodiment of the present invention a birnavirus mutant is provided which can be used not only for the preparation of a vaccine against infection by a specific birnavirus, but also against other poultry or fish infectious diseases. For example, a vector vaccine based on such an IBDV mutant offers the possibility to immunise against other avian pathogens by the expression of antigens of these avian pathogens within infected cells of the immunised host. Such an IBDV vector according to the present invention can be obtained by inserting a heterologous nucleic acid sequence encoding a polypeptide heterologous to the IBDV in the VP5 gene as defined herein.

**[0035]** The heterologous nucleic acid sequence may encode an antigen of an avian pathogen such as Newcastle disease virus, Infectious bronchitis virus, Marek's disease virus, avian encephalomyelitis virus, avian reovirus, avian influenza virus, chicken anaemia virus, *Salmonella* spp., *E. coli*, and *Eimeria* spp.

**[0036]** Furthermore, an IBDV mutant according to the invention comprises in addition to the mutation in the VP5 gene, a mutation in the VP2 gene, wherein this gene expresses a chimeric protein comprising neutralising epitopes of more than one antigenic type of IBDV (e.g. classic, Variant-E and/or GLS). Preferably, such a mutant comprises the relevant protective VP2 epitopes of a variant GLS strain and classic strain. In particular, the mutated VP2 gene is a GLS VP2 gene comprising a nucleic acid sequence fragment encoding the B69 epitope. The construction of such a mutated VP2 genes is described in Snyder et al., Avian Diseases 38, 701-707, 1994. Furthermore, nucleic acid sequences encoding polypeptides for pharmaceutical or diagnostic applications, in particular immuno-modulators such as lymphokines, interferons or cytokines, may be incorporated into the VP5 gene. The heterologous nucleic acid sequence may also encode a screenable marker, such as *E. coli*  $\beta$ -galactosidase or *E. coli*  $\beta$ -glucuronidase.

**[0037]** The construction of birnavirus mutants, in particular of IBDV mutants according to the present invention can be achieved by means of the recently established infectious cRNA system for IBDV (Mundt and Vakharia, Proc. Natl. Acad. Sci. USA 93, 11131-11136, 1996). This reverse genetics system opens the possibility to introduce mutations in the RNA genome of the IBD virus, in particular in the VP5 gene. The most important step in this reverse genetics system is to provide full length cDNA clones of the segments A and B of IBD virus. cDNA constructs comprising the segment A or B, including the nucleotides of the 5'- and 3'- ends of both these segments can be generated according to the method described by Mundt and Vakharia (1996, supra). Additionally, these constructs comprise a RNA polymerase promoter operably linked to either of the segments. The promoter can be the promoter for the T7, SP6 or T3 polymerase, the T7 promoter being preferred. Mutations can be introduced into the VP5 gene by means of methods generally known in the art for this purpose. In particular, the mutation(s) are introduced by means of site directed mutagenesis.

**[0038]** For example, in a first step a cDNA fragment is provided comprising at least a substantial part of the VP5 gene. In the next step suitable primer pairs are designed and hybridised with the VP5 sequence containing fragment. The 5'-primer comprises in addition to sequences complementary to the VP5 sequence, nucleotides which harbour the desired mutation, e.g. a mutation which changes the ATG start codon to an AGG (arginine) codon. Moreover, the 5'-primer is provided with an upstream nucleotide sequence representing a suitable restriction enzyme cleavage site which allows the restoring of the complete 5'-end non-coding sequence. Subsequently, the new mutated fragment is amplified using PCR and the new fragment is introduced in the starting sequence by replacing the native nucleic acid sequence using appropriate restriction enzymes. In the next step plus-sense transcripts of the segment A and B are generated in vitro with (T7) RNA polymerase, after which the synthetic transcripts are purified using conventional RNA purification techniques. The recombinant IBDV mutant according to the invention is obtained after transfection of suitable cells (e.g. VERO cells, QM-7 cells or CEC cells) with the synthetic RNA transcripts of both segments of the IBDV genome, if desired in the presence of transfection-enhancing compositions, such as Lipofectin. Finally the recombinant IBDV is harvested from the supernatant of the transformed cells.

**[0039]** Methods for introducing a mutation in the birnavirus genome are described herein, but are also generally used in the art (Mundt and Vakharia, 1996, supra; Current Protocols in Molecular Biology, eds.: F. M. Ausubel et al., Wiley N.Y., 1995 edition, pages 8.5.1.-8.5.9.)

**[0040]** Further to the unexpected finding by the present inventors that the VP5 ORF of IBDV is a non-essential region of the IBDV genome, it has also been found that an IBDV mutant according to the present invention is able to induce a protective immune response, i.e. animals immunised with a vaccine comprising the IBDV mutant are protected against virulent challenge. Moreover, it has been found that anti-sera of animals infected with naturally occurring IBDV comprise antibodies directed to the non-structural VP5 protein and that these antisera can be distinguished from anti-sera derived from animals infected with an IBDV mutant according to the present invention. In addition, it has been found that the IBDV mutant as described above is attenuated if compared with the parent IBD virus which is able to produce the native VP5 protein.

**[0041]** Therefore, another aspect of this invention is a vaccine for use in the protection of animals against birnavirus infection comprising the birnavirus mutant as characterised above, together with a pharmaceutical acceptable carrier or diluent. In particular, the vaccine according to the invention is a vaccine for use in the protection of poultry against infectious bursal disease comprising the IBDV mutant described above.

**[0042]** The birnavirus mutant according to the present invention can be incorporated into the vaccine as live or inactivated virus.

**[0043]** A vaccine according to the invention can be prepared by conventional methods such as for example commonly used for the commercially available live- and inactivated IBDV vaccines. Briefly, a susceptible substrate is inoculated with an IBDV mutant according to the invention and propagated until the virus replicated to a desired infectious titre after which IBDV containing material is harvested.

**[0044]** Every substrate which is able to support the replication of IBD viruses can be used in the present invention, including primary (avian) cell cultures, such as chicken embryo fibroblast cells (CEF) or chicken kidney cells (CK), mammalian cell lines such as the VERO cell line or the BGM-70 cell line, or avian cell lines such as QT-35, QM-7 or LMH. Usually, after inoculation of the cells, the virus is propagated for 3-10 days, after which the cell culture supernatant is harvested, and if desired filtered or centrifuged in order to remove cell debris.

**[0045]** Alternatively, the IBDV mutant is propagated in embryonated chicken eggs. In particular, the substrate on which these IBD viruses are propagated are SPF embryonated eggs. Embryonated eggs can be inoculated with, for example 0.2 ml IBDV mutant containing suspension or homogenate comprising at least  $10^2$  TCID<sub>50</sub> per egg, and subsequently incubated at 37 °C. After about 2-5 days the IBD virus product can be harvested by collecting the embryo's and/or the membranes and/or the allantoic fluid followed by appropriate homogenising of this material. The homogenate can be centrifuged thereafter for 10 min at 2500 x g followed by filtering the supernatant through a filter (100 µm).

**[0046]** The vaccine according to the invention containing the live virus can be prepared and marketed in the form of a suspension or in a lyophilised form and additionally contains a pharmaceutically acceptable carrier or diluent cus-

tomary used for such compositions. Carriers include stabilisers, preservatives and buffers. Suitable stabilisers are, for example SPGA, carbohydrates (such as sorbitol, mannitol, starch, sucrose, dextran, glutamate or glucose), proteins (such as dried milk serum, albumin or casein) or degradation products thereof. Suitable buffers are for example alkali metal phosphates. Suitable preservatives are thimerosal, merthiolate and gentamicin. Diluents include water, aqueous buffer (such as buffered saline), alcohols and polyols (such as glycerol).

**[0047]** If desired, the live vaccines according to the invention may contain an adjuvant. Examples of suitable compounds and compositions with adjuvant activity are the same as mentioned below.

**[0048]** Although administration by injection, e.g. intramuscular, subcutaneous of the live vaccine according to the present invention is possible, the vaccine is preferably administered by the inexpensive mass application techniques commonly used for IBDV vaccination. For IBDV vaccination these techniques include drinking water and spray vaccination.

**[0049]** Alternative methods for the administration of the live vaccine include in ovo, eye drop and beak dipping administration.

**[0050]** In another aspect of the present invention a vaccine is provided comprising the birnavirus mutant in an inactivated form. The major advantage of an inactivated vaccine is the extremely high levels of protective antibodies of long duration that can be achieved.

**[0051]** The aim of inactivation of the viruses harvested after the propagation step is to eliminate reproduction of the viruses. In general, this can be achieved by chemical or physical means. Chemical inactivation can be effected by treating the viruses with, for example, enzymes, formaldehyde,  $\beta$ -propiolactone, ethylene-imine or a derivative thereof. If necessary, the inactivating compound is neutralised afterwards. Material inactivated with formaldehyde can, for example, be neutralised with thiosulphate. Physical inactivation can preferably be carried out by subjecting the viruses to energy-rich radiation, such as UV light or  $\gamma$ -rays. If desired, after treatment the pH can be adjusted to a value of about 7.

**[0052]** A vaccine containing the inactivated birnavirus mutant can, for example comprise one or more of the above-mentioned pharmaceutically acceptable carriers or diluents suited for this purpose.

**[0053]** Preferably, an inactivated vaccine according to the invention comprises one or more compounds with adjuvant activity. Suitable compounds or compositions for this purpose include aluminium hydroxide, -phosphate or -oxide, oil-in-water or water-in-oil emulsion based on, for example a mineral oil, such as Bayol F® or Marcol 52® or a vegetable oil such as vitamin E acetate, and saponins.

**[0054]** The vaccine according to the invention comprises an effective dosage of the birnavirus mutant as the active component, i.e. an amount of immunising birnavirus material that will induce immunity in the vaccinated birds against challenge by a virulent virus. Immunity is defined herein as the induction of a significant higher level of protection in a population of birds after vaccination compared to an unvaccinated group.

**[0055]** Typically, the live vaccine according to the invention can be administered in a dose of  $10^2$ - $10^9$  TCID<sub>50</sub> infectious dose<sub>50</sub> (TCID<sub>50</sub>) per animal, preferably in a dose ranging from  $10^{5.0}$ - $10^{7.0}$  TCID<sub>50</sub>, and an inactivated vaccines may contain the antigenic equivalent of  $10^5$ - $10^9$  TCID<sub>50</sub> per animal.

**[0056]** Inactivated vaccines are usually administered parenterally, e.g. intramuscularly or subcutaneously.

**[0057]** Although, the IBDV vaccine according to the present invention may be used effectively in chickens, also other poultry such as turkeys, guinea fowl and partridges may be successfully vaccinated with the vaccine. Chickens include broilers, reproduction stock and laying stock.

**[0058]** The age of the animals receiving a live or inactivated vaccine according to the invention is the same as that of the animals receiving the conventional live- or inactivated IBDV vaccines. For example, broilers (free of maternally derived antibodies-MDA) may be vaccinated at one-day-old, whereas broilers with high levels of MDA are preferably vaccinated at 2-3 weeks of age. Laying stock or reproduction stock with low levels of MDA may be vaccinated at 1-10 days of age followed by booster vaccinations with inactivated vaccine on 6-8 and 16-20 weeks of age.

**[0059]** The invention also includes combination vaccines comprising, in addition to the IBDV or IPNV mutant according to the invention, one or more immunogens derived from other pathogens infectious to poultry or fish, respectively.

**[0060]** Preferably, the combination vaccine additionally comprises one or more vaccine strains of infectious bronchitis virus (IBV), Newcastle disease virus (NDV), egg drop syndrome (EDS) virus, turkey rhinotracheitis virus (TRTV) or reovirus.

**[0061]** In addition to a marker vaccine for birnaviruses, the availability of an appropriate diagnostic test is an essential requirement for the application of a birnavirus eradication control programme. Such a diagnostic test is provided herein and comprises a method for determining IBDV infection in poultry and IPNV infection in fish, i.e. it provides a method for distinguishing an animal in the field vaccinated with a vaccine as described above, from an animal infected with a naturally-occurring IBDV or IPNV.

**[0062]** Therefore, the present invention provides a method for the detection of birnavirus infection, in particular for the detection of IBDV infection in an animal comprising the step of examining a sample of the animal for the presence of VP5 antibodies or antigens. The animal is an animal from the field and is in particular an avian species, preferably

a chicken. The sample coming from the animal may be any sample in which IBDV antibodies or antigens are present, e.g. a blood, serum or tissue sample, the serum sample being preferred.

**[0063]** A preferred method for determining birnavirus infection in an animal is a method for the detection of antibodies against the VP5 protein, comprising the steps of:

- (i) incubating a sample suspected of containing anti-birnavirus antibodies, with VP5 antigen,
- (ii) allowing the formation of antibody-antigen complex, and
- (ii) detecting the presence of the antibody-antigen complex.

**[0064]** The design of this immunoassay may vary. For example, the immunoassay may be based upon competition or direct reaction. Furthermore, protocols may use solid supports or may use cellular material. The detection of the antibody-antigen complex may involve the use of labelled antibodies; the labels may be, for example, enzymes, fluorescent-, chemiluminescent-, radio-active- or dye molecules.

**[0065]** Suitable methods for the detection of the VP5 antibodies in the sample include the enzyme-linked immunosorbent assay (ELISA), immunofluorescent test (IFT) and Western blot analysis.

**[0066]** In an exemplifying ELISA, the wells of a polystyrene micro-titration plate are coated with VP5 antigen. Next, the wells of the coated plates are filled with chicken serum and serial dilutions are made. After incubation, chicken anti-VP5 protein serum antibodies are determined by detecting antibody (monoclonal or polyclonal) with the same specificity as the coated one, but which is labelled (e.g. with biotin). The labelled antibody will occupy the free antigens that have not been occupied by anti-VP5 antibodies in the chicken serum. For example, horse radish peroxidase coupled to avidin may be added and the amount of peroxidase is measured by an enzymatic reaction. If no antibodies against VP5 are present in the chicken serum sample then a maximum absorption is obtained. If the serum contains many antibodies against VP5 then a low absorption is expected. Alternatively, after the incubation with chicken serum, the amount of antibodies present in the serum that bound to the VP5 antigen may be determined directly by using an anti-chicken conjugate followed by the enzymatic reaction.

**[0067]** In a sandwich ELISA the wells of a polystyrene micro-titration plate can be coated with a monoclonal antibody directed against the VP5 protein. Next, the wells of these coated plates are incubated with VP5 antigen. After the antigen is captured, the wells are filled with the chicken serum and serial dilutions are made. Subsequently, the protocol as described above may be followed. This test can also be carried out by using polyclonal serum against VP5 instead of the coated monoclonal antibodies.

**[0068]** In another diagnostic test (Western blot analysis), the VP5 antigen (containing) material is subjected to SDS-PAGE. Next, the separated proteins are electroblotted onto nitro-cellulose membrane. Thereafter, the membranes can be cut into lanes and the lanes are incubated with the chicken serum. The presence of VP5 antibodies in the sample can be determined by examination whether antibodies bound to the VP5 antigen, for example by using an anti-chicken conjugate followed by an enzymatic reaction. If antibodies against VP5 are present then a band at about 17 kDa is identifiable.

**[0069]** The VP5 antigen may be any VP5 protein (fragment) comprising material which allows the formation of the VP5 antigen-VP5 antibody complex. Preferably, the VP5 antigen comprises the expression product of a conventional recombinant host cell or virus, e.g. such as E.coli expressed VP5 (Mundt et al., J. Gen. Virol. 76, 437-443, 1995) or baculovirus expressed protein (Vakharia et al., Vaccine 12, 452-456, 1994; Vakharia et al., J. Gen Virol. 74, 1201-1206, 1993). In a further embodiment of the present invention a diagnostic test kit is provided which is suitable for performing the diagnostic test according to the invention as described above.

**[0070]** In particular, a diagnostic test kit is provided which comprises in addition to the components usually present, the VP5 antigen (if desired coated onto a solid phase) as the immunological reagent. Other components usually present in such a test kit include, biotin or horseradish peroxidase conjugated antibodies, enzyme substrate, washing buffer etc.

**[0071]** To determine birnavirus VP5 antigen in a test sample from an animal in the field, VP5-specific antibodies are used as the immunological reagent, preferably fixed to a solid phase. The test sample is added, and after an incubation time allowing formation of the antibody-antigen complex, a second labelled antibody may be added to detect the complex.

## EXAMPLES

### Example 1.

#### Construction and analysis of recombinant VP5<sup>-</sup> IBD virus

##### Construction of full length VP5<sup>-</sup> clone of IBDV segment A.

[0072] To construct a VP5-negative IBDV, the *EcoRI* site immediately following the 3'-end of the full length cDNA of strain D78 segment A (pUC19FLAD78; Mundt and Vakharia, Proc. Natl. Acad. Sci. USA 93, 11131-11136, 1996) was deleted. An *EcoRI* - *KpnI* fragment containing the T7 polymerase binding site followed by the complete segment A sequence was excised and inserted into *EcoRI* - *KpnI* cleaved vector pUC18 after inactivation of the unique *NdeI* within the vector sequence resulting in plasmid pAD78/EK. Thereafter, the genomic region encompassing the initiation codon for VP5 was amplified in two pieces using primers A1F5' and VP5MutR, and VP5MutF and A2R, respectively (see Table 1 for sequence and location of primers). PCR fragments were cloned separately and were subsequently fused via a unique *AflI* site which had been created by mutations within respective primers (see Fig. 2). An *EcoRI* - *NdeI* fragment containing the T7 polymerase binding site, and the 5'-part of segment A including the introduced mutations was excised and used to substitute the wild-type *EcoRI* - *NdeI* fragment in pAD78/EK to yield plasmid pAD78/VP5<sup>-</sup>. Of the three mutations introduced one altered the initiation methionine codon for VP5 into an arginine codon (Fig. 2).

Table 1: Sequence of oligonucleotide primers used for generating mutant constructs.

aNucleotide sequence	Orientation	Designation	Nucleotide no.
AGAGAAATTCTAATACGACTCACTATAGGA TACGATCGGTCTGAC	+	A1F5'	1-18
TGGGCTGTCACTGCTGTCACATGT	-	A2R	716 - 740
CATTGCTCTGCAGTGTGTAGTGAGC	-	A3R	338 - 362
CTACAACGCTATCCTTAAGGGTTAGTA GAG	+	VP5MutF	80 - 109
CTCTACTAACCCTTAAGGATAGCGTTGT AG	-	VP5MutR	80 - 109

- a) Underlined nucleotides denote virus specific nucleotides. T7 promotor sequences are marked in italics. Mutated nucleotides are bold and orientation of the primer is shown for sense (+) and antisense (-). Primer positions are given according to the published sequence of serotype I strain P2 (Mundt et al., Virology 209, 209-218, 1995).

[0073] **Virus recovery from cRNA.** For *in vitro* transcription of RNA plasmids pAD78/EK, pAD78/VP5<sup>-</sup> and pBP2 (Fig. 2) were linearized by cleavage with *BsrGI* and *PstI*, respectively. Treatment of linearized DNA, transcription and purification of RNA, and transfection were carried out as described by Mundt and Vakharia (1996, *supra*) with the exception that secondary CEC were used for the transfection experiments. Three days after transfection a CPE was visible in CEC. Cells were freeze/thawed, centrifuged at 700 x g to eliminate cellular debris, and the resulting supernatants were filtrated through 0.45 µm filters and stored at -20°C. For the transfection experiments full length cDNA clones of segment A of strain D78 capable of expressing (pAD78/EK) or unable to express VP5 (pAD78/VP5<sup>-</sup>) were transcribed into synthetic RNA and cotransfected with segment B full length cRNA into CEC. Resulting virus progeny IBDV/EK and IBDV/VP5<sup>-</sup> was further characterised.

[0074] **Analysis of transfection progeny by immunofluorescence and Radioimmunoprecipitation assay**



(RIPA). VP5 was expressed in E.coli as described in Mundt et al. (J. Gen. Virol. 76, 437-443, 1995). Rabbit monospecific polyclonal anti serum and mouse monoclonal antibodies against VP5 were prepared according to standard protocols. Vero cells infected with IBDV/VP5<sup>-</sup>, IBDV/EK, and non-infected cells, respectively, were incubated with rabbit anti-IBDV serum, rabbit anti-VP5 serum and with anti-VP5 mAb DIE 7, and stained with fluoresceine-conjugated secondary antibodies. Both antisera and the monoclonal antibody recognised IBDV antigens in the cytoplasm of IBDV/EK infected cells. In contrast, whereas the anti-IBDV serum readily detected viral antigens in IBDV/VP5<sup>-</sup> infected cells, neither the monospecific anti VP5-serum nor the monoclonal anti-VP5 antibody exhibited specific reactivity. None of these immunological reagents reacted with non-infected controls.

[0075] To analyse viral proteins expressed during replication lysates of radioactively labelled CEC infected with IBDV/VP5<sup>-</sup> (Fig 4, lanes 1-3) and IBDV/EK (Fig. 4, lanes 4-6) were immunoprecipitated with rabbit anti-IBDV serum, rabbit anti-VP5 serum and mAb DIE 7. Non-infected CEC were used as control (Fig. 4, lanes 7-9). IBDV/EK (lane 4) as well as IBDV/VP5<sup>-</sup> (lane 1) infected CEC showed viral proteins VP2, VP3, and VP4 after precipitation with rabbit anti-IBDV serum. The rabbit anti-VP5 serum (lane 5) and mAb DIE 7 (lane 6) precipitated VP5 with a molecular mass of 21 kDa only from IBDV/EK infected cells. No specific reactivity was detectable in IBDV/VP5<sup>-</sup> infected CEC after precipitation with rabbit-anti VP5 (lane 2) as well as the VP5 specific mAb DIE 7 (lane 3). Non-infected CEC showed no specific reactivity (lanes 7-9).

[0076] **Replication of IBDV/VP5<sup>-</sup> in CEC.** To assay replication of IBDV/VP5<sup>-</sup> in more detail one step growth was analysed (Fig. 5). Confluent secondary CEC were infected with IBDV/EK and IBDV/VP5<sup>-</sup> with  $10^{7.2}$  TCID<sub>50</sub>, respectively. Immediately after overlaying the infected cells with 5 ml growth medium, supernatant from one infected CEC tissue plate of each virus was removed and stored at -20°C (0 h p.i.). Remaining tissue culture plates were further incubated and 4h, 8h, 16h, 24h, and 48h p.i. supernatants were removed and stored at -20°C. Supernatants were centrifuged and titrated according to standard methods. The TCID<sub>50</sub> at the different time points after infection showed that the VP5 expressing virus (IBDV/EK) replicated faster than the virus mutant lacking VP5 (IBDV/VP5<sup>-</sup>). 16 h after infection IBDV/EK showed a 100-fold higher than IBDV/VP5<sup>-</sup> (Fig. 5). However, at 48 h p.i. IBDV/VP5<sup>-</sup> reached a titre of  $10^{7.2}$  TCID<sub>50</sub>/ml which was similar to IBDV/EK ( $10^{7.45}$ /ml)

[0077] **Preparation of recombinant IBDV VP5-2.** Plasmid pAD78/VP5-2 was prepared by techniques similar to those described above. The nucleotide sequence of part of the mutated VP5 gene is shown in SEQ ID No. 7 and Figure 3. A restriction enzyme fragment harbouring the mutations was used to substitute the wild-type *EcoRI* - *NdeI* fragment in pAD78/EK. An outline of the protocol for the preparation of the recombinant plasmid is shown in Figure 3. The organisation of pBD78 is also depicted in Figure 3. The recombinant virus was prepared as described above, except for the fact that segment B of strain D78 (SEQ ID No. 8) was used and QM-7 cells were used for the transfection experiment.

## Example 2

### Identification of VP5 protein in different IBDV strains

[0078] Different strains of IBDV were investigated for the expression of the VP5-gene. This was done by making use of the immuno-fluorescence technique (IFT). Chicken embryo fibroblasts grown in microtiterplates were infected with different IBDV strains. Three to 5 days after incubation at 37°C cells were fixed with 70% ethanol, then treated with polyclonal rabbit anti IBDV serum (R1928), polyclonal rabbit anti VP5 serum (RαVP5) or monoclonal antibody directed against VP5 (DIE7), respectively. Binding of the poly- or monoclonal antibodies to the different IBDV strains was visualised by making use of a fluorescence labelled conjugate (goat-anti-rabbit or goat-anti-mouse). The results are shown in Table 2:

Table 2

Identification of different sero- and subtypes of IBDV strains. Determination of the presence of VP5 proteins.					
IBDV-serotype	IBDV-subtype	IBDV-strain	R1928	RαVP5	DIE7
I	Classical	D78	+	+	+
I	Classical	228TC	+	+	+
I	Classical	PBG98	+	+	+
I	Classical	Ram0404	+	+	+
I	Classical	IBDV/EK	+	+	+
I	Classical	IBDV/VP5 <sup>-</sup>	+	-	-

Table 2 (continued)

Identification of different sero- and subtypes of IBDV strains. Determination of the presence of VP5 proteins.					
IBDV-serotype	IBDV-subtype	IBDV-strain	R1928	RαVP5	DIE7
I	GLS	GLS	+	+	+
I	Variant-E	8903	+	+	+
II	TY89	TY89	+	+	+

[0079] From these data it can be concluded that the different strains of IBDV belonging to different sero- and subtypes do express the VP5-gene. Furthermore, the recombinant VP5<sup>-</sup> IBDV vaccine strain can be differentiated from field and vaccine viruses, thereby enabling the recombinant VP5<sup>-</sup> virus to be used as a marker vaccine.

### Example 3

In vivo testing of the recombinant VP5<sup>+</sup> and VP5<sup>-</sup> IBDV vaccines in comparison with a commercial available live IBDV vaccine.

[0080] **Preparation of IBDV vaccine.** Primary chicken embryo fibroblast (CEF) cells were prepared at a final concentration of  $2 \times 10^6$ /ml. The cells were cultured in Eagles minimum essential medium containing 5% fetal calf serum. To 25 ml of this cell suspension 0.1 ml IBDV/EK or IBDV/VP5<sup>-</sup> virus (having an infectious titre of about  $3.0 \log_{10}$  TCID<sub>50</sub>/ml) was added. After incubation for 5 days in a high-humidity incubator at 37°C, the total suspension was used in the animal experiment without further purification. The infectious titre of the supernatant was  $10^{7.1}$  TCID<sub>50</sub>/ml.

[0081] **Animal experiment.** In this study the potency of different vaccines (VP5 positive strain IBDV/EK and a VP5 negative strain IBDV/VP5<sup>-</sup>, and the commercial available IBDV vaccine Nobilis strain D78, Intervet International B.V., NL) was investigated. SPF chicks of 3 weeks old were treated as indicated in the treatment schedule.

Treatment Schedule:

Days after vaccination	Groups			
	1	2	3	4
00	IBDV/EK	IBDV/VP5 <sup>-</sup>	D78	-
03	x	x1	x	x
07	x,bl	x1,bl	x,b	x,bl
14	x,bl	x,bl	x,bl	x,bl
20	x,bl	x,bl	x,bl	x,bl
21	ch	ch	ch	ch
24	x	x	x	x
31	+	+	+	+
VP5 <sup>+</sup> Bursal disease vaccination with VP5 positive vaccine clone, eye-drop route, dose $10^{4.6}$ TCID <sub>50</sub> /animal, 0.1 ml/animal. VP5 <sup>-</sup> Bursal disease vaccination with VP5 negative vaccine clone, eye-drop route, dose $10^{5.9}$ TCID <sub>50</sub> /animal, 0.1 ml/animal. D78 Bursal disease vaccination with IBDV VACCINE NOBILIS STRAIN D78, eye-drop route, one field dose. ch Challenge with Bursal disease virus, Farragher strain F52/70, eye-drop route, dose $10^{2.0}$ CID <sub>50</sub> /animal, 0.1 ml/animal. bl Serological examination; VN-test and/or Western blotting. x Histological examination (H.E. staining) and MCA-8 ELISA on bursae. x1 Histological examination (H.E. staining) and MCA-8 ELISA on bursae and reisolation of virus from bursa of Fabricius. + Clinical examination and after 10 days histological examination of the bursa.				

# **Detection of virus in the bursa of Fabricius.**

**[0082]** Three, 7, 14 and 20 days after eye-drop vaccination, animals were sacrificed and blood and bursae obtained. The presence of virus in the bursa was determined with an enzyme-linked immunosorbent assay (ELISA) making use of the monoclonal antibody 8 (MAB-8). MAB-8 is directed specifically against IBDV. Data are depicted in Table 3.

**[0083]** Furthermore, 3 and 7 days after vaccination, bursae from animals of group 2 were investigated for the presence of the recombinant VP5<sup>-</sup> virus. For that purpose bursae were homogenised and cultured on chicken embryo fibroblasts. The presence of the VP5<sup>-</sup> virus was determined by IFT using polyclonal rabbit sera against IBDV or VP5 or monoclonal antibodies against VP5. From 13 out of 15 bursae (87%) investigated, VP5<sup>-</sup> virus could be reisolated and identified (positive for R1928 and negative for RαVP5 and DIE7). This indicates that the virus upon animal passage is still VP5<sup>-</sup>, indicating that the virus is stable and does not revert to VP5<sup>+</sup>. Furthermore, by using the different poly- and monoclonal antibodies VP5<sup>-</sup> vaccine virus can be discriminated from all other vaccine and/or field IBDV viruses. Therefore, the VP5<sup>-</sup> vaccine may be used as a marker vaccine.

**[0084]** Three days after challenge no virus could be detected in groups 1, 2 and 3 with the MCA-8 ELISA. In contrast, all animals of group 4 (non-vaccinated control group) contained challenge virus in the bursa of Fabricius, 3 days after challenge. The results show that animals vaccinated with recombinant VP5<sup>+</sup> (group 1), recombinant VP5<sup>-</sup> (group 2) and IBDV vaccine Nobilis D78 (group 3) were protected against severe challenge.

Table 3

Individual data for detection of virus in the bursa of Fabricius with the MCA-8 ELISA at different days after vaccination or challenge.						
	Days after vaccination→				Days after challenge	
	3	7	14	20	3	
Group↓	Virus detection by ELISA↓					Protection↓
1 VP5 <sup>+</sup>	2/8*	1/7	0/2	0/3	0/5	100%
2 VP5 <sup>-</sup>	0/8	0/7	0/2	0/3	0/5	100%
3 D78	1/8	6/7	0/2	0/3	0/5	100%
4 -	0/8	0/7	0/2	0/3	5/5	0%

\*Number of positive bursae per total number tested.

# **Detection of lesions in the bursa of Fabricius.**

**[0085]** The microscopic average lesion score induced by the different IBDV (recombinant) vaccines or the challenge virus are depicted in Table 4.

**[0086]** Before challenge, animals vaccinated with the recombinant VP5<sup>+</sup> IBDV vaccine (group 1) or vaccinated with IBDV vaccine Nobilis D78 (group 3) showed mild to moderate lesions in the bursa. Three days after challenge only chronic lesions were observed in the bursa of Fabricius, indicating that the animals of groups 1 and 3 were protected against challenge. Furthermore, 10 days after challenge only very mild lesions (0-20% lymphocytic depletion) were observed in the bursa of the animals vaccinated with VP5<sup>+</sup> recombinant IBDV vaccine or with Nobilis vaccine D78. In contrast animals not vaccinated and challenged showed severe lesions 10 days after challenge. In other words all animals (100%) of groups 1 and 3, vaccinated with the VP5<sup>+</sup> recombinant IBDV vaccine or with Nobilis vaccine D78 were protected against severe challenge.

**[0087]** Three, 7, 14 and 20 days after vaccination and 3 and 10 days after challenge with the recombinant VP5<sup>-</sup> IBDV vaccine, animals of group 2 showed no to hardly any lesions (0-20% lymphocytic depletion) in the bursa. All animals of group 2, vaccinated with the VP5<sup>-</sup> recombinant IBDV vaccine, were protected against severe challenge. When animals vaccinated with the recombinant VP5<sup>-</sup> IBDV vaccine are compared to animals of groups 1 or 3 (vaccinated with a recombinant VP5<sup>+</sup> or commercial available vaccine) the recombinant VP5<sup>-</sup> vaccine induces less lesions and therefore, is safer, milder than the vaccines tested in this experiment.

**[0088]** Three days post-challenge, all non-vaccinated animals of group 4 showed severe acute lesions in the bursa (total lymphocyte depletion, score 5.0). Ten days after challenge, all animals (17 out of 17 animals) showed total lymphocytic depletion, indicating that these animals were not protected against severe challenge. Animals that died after challenge, all showed severe lesions in the bursa of Fabricius. It was concluded that control group 4 was not protected against severe challenge indicating that the test conditions were optimal.

Table 4

Average bursal lesion score at different days after vaccination or challenge. The average lesion score is calculated as follows: all lesion scores from the animals per group on a certain day are added. This number is then divided by the total number of animals investigated in that group on that day. Individual scores range from 1 to 5. Score 0 = no lymphocytic depletion, score 1 = 0 - 20%; score 2 = 20 - 40%; score 3 = 40 - 60%; score 4 = 60 - 80% and score 5 = 80 - 100 % lymphocytic depletion (total lymphocytic depletion).

	Days after vaccination→				Days after challenge→		
	3	7	14	20	3	10	
Group↓	Bursal lesions score↓						Protection↓
1 VP5 <sup>+</sup>	0.8	2.9	1.0	1.0	1.0 <sup>c</sup>	0.6	100%
2 VP5 <sup>-</sup>	0.0	0.0	0.5	0.0	0.0 <sup>c</sup>	0.1	100%
3 D78	0.1	2.4	3.5	2.0	2.8 <sup>c</sup>	1.1	100%
4 -	0.0	0.0	0.0	0.0	5.0 <sup>a</sup>	5.0	0%

<sup>a</sup> Acute lesions<sup>c</sup> Chronic lesions

### Serological response.

[0089] The serological response of the animals was determined by measuring the ability of blood serum to neutralise a classical infectious bursal disease virus strain in a virus neutralising (VN) test. Serum was investigated 3, 7, 14 and 20 days after vaccination. The average neutralising titres are shown in Table 5.

[0090] The results show that recombinant IBDV vaccine VP5<sup>+</sup> applied to chickens of group 1 induced a good and high serological response 20 days after vaccination which is comparable to the serological response of the chickens vaccinated with the commercial IBDV vaccine Nobilis strain D78 (group 3). The recombinant IBDV vaccine VP5<sup>-</sup> applied to chickens of group 2 induced also a good serological response. A titre of 9.4 log<sub>2</sub> was observed 20 days after vaccination. The serological response induced by the recombinant VP5<sup>-</sup> IBDV vaccine was delayed when compared to the serological response induced by the recombinant IBDV VP5<sup>+</sup> vaccine or the commercial IBDV vaccine Nobilis strain D78.

[0091] The non-vaccinated group 4 showed no serological response to IBDV.

Table 5

Average IBDV-VN-titres for groups 1 to 4 at different days after vaccination, expressed as log<sub>2</sub> of the dilution.

Group	Days after vaccination			
	3	7	14	20
1 VP5 <sup>+</sup>	≤ 1.0 ± 0.0	7.1 ± 1.7	10.2 ± 1.4	11.9 ± 1.8
2 VP5 <sup>-</sup>	≤ 1.0 ± 0.0	2.1 ± 1.7	6.3 ± 2.9	9.4 ± 1.4
3 D78	≤ 1.0 ± 0.0	5.2 ± 2.8	10.3 ± 1.3	11.6 ± 1.5
4-	≤ 1.0 ± 0.0	≤ 1.0 ± 0.0	≤ 1.0 ± 0.0	≤ 1.0 ± 0.0

### Serological differentiation between antisera.

[0092] The serological response against VP5 was investigated by making use of western blot analysis. For this purpose the VP5 protein was expressed in the E. coli or baculo expression system. The expressed proteins were separated by SDS PAGE. Next the proteins were electroblotted onto a nitro-cellulose membrane. Thereafter, the membrane was cut into lanes and the lanes were incubated with rabbit anti-VP5 serum, chicken serum directed against VP5<sup>+</sup> recombinant vaccine, chicken serum directed against VP5<sup>-</sup> recombinant vaccine or negative serum from SPF chickens. Data are summarised in Table 6. As can be seen from Table 6, the VP5<sup>-</sup> serum does not induce a serological response against VP5. In contrast the rabbit anti-VP5 serum and chicken serum directed against VP5<sup>+</sup> recombinant vaccine do recognise the VP5-protein and thus induces a serological response against VP5. This indicates that chicken serum may be used to investigate if animals are exposed to a virus that expresses the VP5 protein (e.g. field virus) or

to the VP5<sup>+</sup> recombinant vaccine.

Table 6

Western blot analysis. Serum from animals vaccinated with VP5 <sup>+</sup> or VP5 <sup>-</sup> recombinant vaccine as well as SPF chicken serum and anti VP5-rabbit serum were investigated for their reaction with the VP5-protein.	
Identification of serum sample	Immuno-blot
VP5 <sup>+</sup> vaccinated animal, serum sample 20d after vaccination	positive
VP5 <sup>-</sup> vaccinated animal, serum sample 20d after vaccination	negative
Non-vaccinated control, serum sample at 20d	negative
Rabbit anti VP5 serum	positive

#### Mortality and clinical signs.

[0093] None of the animals vaccinated with VP5<sup>+</sup> IBDV vaccine (group 1), vaccinated with recombinant VP5<sup>-</sup> IBDV vaccine (group 2) or vaccinated with the commercial IBDV vaccine Nobilis strain D78 (group 3), died or showed clinical signs of infectious bursal disease after challenge, indicating that the animals were protected against severe challenge. All animals in the non-vaccinated control group were not protected against severe challenge.

#### Example 4

##### In vivo testing of the recombinant VP5-2 vaccine

[0094] **Preparation of the IBDV vaccines.** Primary chicken embryo fibroblasts (CEF) cells were prepared at a final concentration of  $2 \times 10^6$ /ml. The cells were cultured in Eagles minimum essential medium containing 5% fetal calf serum. To 15 ml of this cell suspension 0.1 ml IBDV/VP5-2 (D78/D78/VP5<sup>-</sup>) virus was added. After incubation for 6 days in a high humidity incubator at 37°C, the supernatant was titrated. The infectious titre of the supernatant was  $10^{8.2}$  TCID<sub>50</sub>/ml. For the second animal experiment the supernatant was diluted to result in a vaccine dose of  $10^{5.5}$  TCID<sub>50</sub>/animal and for the first animal experiment the supernatant was diluted to result in a vaccine dose of  $10^{4.0}$  TCID<sub>50</sub>/animal or  $10^{5.0}$  TCID<sub>50</sub>/egg.

[0095] **First animal experiment.** The effect of the vaccine is assessed by measurement of the serological response and resistance to challenge obtained from administering a challenge virus at the age of 14 days. The vaccine ( $10^{5.0}$  TCID<sub>50</sub>/egg or  $10^{4.0}$  TCID<sub>50</sub>/animal of D78/D78/VP5<sup>-</sup>) was applied *in ovo* or intramuscularly at day old. Microscopic lesions in the bursa were investigated, 3 and 10 days after challenge. Protection against challenge was determined and the serological response at the age of 14 days old was determined with the VN-test.

1. Average microscopic lesion score in the bursa 3 and 10 days after challenge.

Days post	Group		
challenge	<i>In ovo</i>	Day old	None-vaccinated
3	3.3	0.0	5.0
10	0.2	0.0	5.0

## 2. Protection after challenge

	Group		
	<i>In ovo</i>	Day old	None-vaccinated
% protection	91.6	100	0

## 3. Serological response against IBDV

	Group		
	<i>In ovo</i>	Day old	None-vaccinated
VN-titre	6.4 ± 1.7	6.4 ± 1.3	<4.0 ± 0.0

VN-titre is expressed as log<sub>2</sub> of the dilution. Animals with a titre <4.0 log<sub>2</sub> are considered negative

## Conclusions

## [0096]

- 1 The D78/D78/VP5<sup>-</sup> strain is a highly attenuated IBD-virus
- 2 The virus strain is very mild
- 3 The virus can induce a serological response
- 4 The virus can induce protection
- 5 The virus strain can be applied by intramuscular injection to 1 day old SPF chickens and *in ovo* to 18-days-old embryonated SPF-eggs

**[0097] Second animal experiment.** The effect of the vaccine is assessed by measurement of the serological response against IBDV and resistance to challenge obtained from administering a challenge virus, 21 days after administering the Gumboro vaccine. The vaccine (10<sup>5.5</sup> TCID<sub>50</sub>/animal of D78/D78/VP5<sup>-</sup>) was applied via the intramuscular route to 14 days old SPF-chickens. Three, 7, 14, and 20 days after vaccination and 3 days after challenge Bursa, spleen, thymus, liver, duodenum, pancreas, ceecal tonsils and harderian gland were investigated for microscopic lesions. Ten days after challenge Bursae were investigated for microscopic lesions. Sera were tested in the VN-test. And mortality was scored after challenge.

## 1. Percentage mortality after challenge:

	Mortality after challenge
Vaccinated group	0%
Control group	50%

## 2. Microscopic lesions of the vaccinated group before and after challenge:

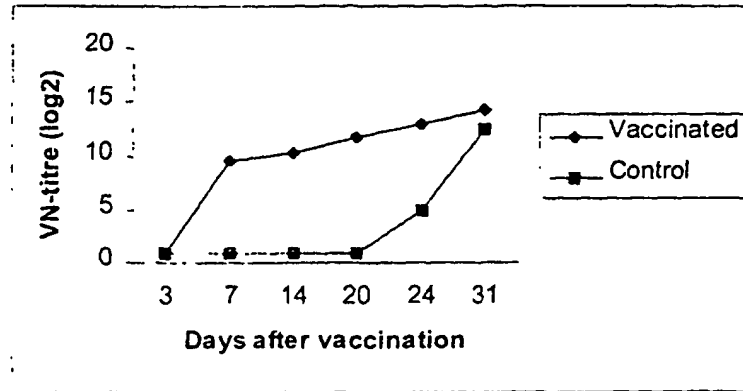
Days post	Bursa	Spleen	Thymus	Liver	Duodeum	Pancreas	Cecal	Harderian
Vaccinat.							Tonsils	Gland
3	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0

(continued)

2. Microscopic lesions of the vaccinated group before and after challenge:								
Days post Vaccinat.	Bursa	Spleen	Thymus	Liver	Duodeum	Pancreas	Ceecal Tonsils	Harderian Gland
14	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0
24	0,A	0	0	0	0	0	0	0
31	0,A	ND	ND	ND	ND	ND	ND	ND

A = None vaccinated animals showed a lymphocytic depletion score of 5.0 (100%) and 4.25, 3 and 10 days after challenge, respectively. ND = not done.

### 3. Serological response after vaccination:



### Conclusions

[0098]

1. The D78/D78/VP5<sup>-</sup> strain is a highly attenuated IBD-virus
2. The virus strain is very mild and does not induce lesions in organs
3. The virus can induce a serological response
4. The virus can induce protection

### LEGENDS TO THE FIGURES

[0099] Figure 1 Genomic organization of segment A and segment B of IBDV. The numbers indicate the nucleotide positions of the start, end and coding region on the segments.

[0100] Figure 2 Construction of genomic cDNA clones for the preparation of IBDV/VP5<sup>-</sup>. Plasmid pAD78/EK contains the complete D78 segment A cDNA encoding the polyprotein (VP2-VP4-VP3) and VP5. Plasmid pBP2 contains the complete strain P2 segment B encoding VP1. Mutations were introduced in plasmid pAD78/VP5<sup>-</sup> altering the methionine start codon for VP5 into arginine and creating an artificial Afl II cleavage site. Recombinant plasmids were linearized with the underlined restriction enzymes, followed by T7 polymerase transcription.

[0101] Figure 3 Construction of genomic cDNA clones for the preparation of IBDV/VP5<sup>-</sup>2. Plasmid pAD78/EK contains the complete D78 segment A cDNA encoding the polyprotein (VP2-VP4-VP3) and VP5. Plasmid pBD78 contains the complete strain D78 segment B encoding VP1. Mutations were introduced in plasmid pAD78/VP5<sup>-</sup> altering the methionine start codon for VP5 into glutamic acid and creating an artificial BstBI cleavage site. Further mutations were introduced in the arginine and glutamine codon. Recombinant plasmids were linearized with the underlined restriction

enzymes, followed by T7 polymerase transcription.

**[0102]** Figure 4 Radioimmunoprecipitation of proteins from CEC infected cells with recombinant IBDV. CEC infected cells with IBDV/VP5<sup>-</sup> (lanes 1-3), IBDV/EK (lanes 4-6) and uninfected controls were immunoprecipitated with rabbit anti-IBDV serum (lanes 1, 4, 7), rabbit anti-VP5 serum (lanes 2, 5, 8) and mAb DIE 7 (lanes 3, 6, 9). Position of molecular mass markers (M) is indicated. Location of the viral proteins VP2, VP3, VP4 and VP5 are marked.

**[0103]** Figure 5 Replication kinetics of IBDV/EK and IBDV/VP5<sup>-</sup>. Infectious titers of supernatants (vertical axis) are determined at the times indicated.

## SEQUENCE LISTING

### **[0104]**

#### (1) GENERAL INFORMATION:

##### (i) APPLICANT:

- (A) NAME: Azko Nobel N.V.
- (B) STREET: Velperweg 76
- (C) CITY: Arnhem
- (E) COUNTRY: The Netherlands
- (F) POSTAL CODE (ZIP): 6824 BM
- (G) TELEPHONE: 0412 666379
- (H) TELEFAX: 0412 650592

##### (ii) TITLE OF INVENTION: Recombinant birnavirus vaccine

##### (iii) NUMBER OF SEQUENCES: 8

##### (iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: Patent In Release #1.0, Version #1.30 (EPO)

#### (2) INFORMATION FOR SEQ ID NO: 1:

##### (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2827 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

##### (ii) MOLECULE TYPE: cDNA

##### (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 112..2745

##### (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GGATACGATG GGTCTGACCC TCTGGGAGTC ACGAATTAAC GTGGCTACTA GGGGCGATAC



EP 0 887 412 B1

	CCGCCGCTGG CCGCCACGTT AGTGGCTCCT CTTCTTGATG ATTCTGCCAC C ATG AGT	117
	Met Ser	
	1	
5		
	GAC ATT TTC AAC AGT CCA CAG GCG CGA AGC ACG ATC TCA GCA GCG TTC	165
	Asp Ile Phe Asn Ser Pro Gln Ala Arg Ser Thr Ile Ser Ala Ala Phe	
	5 10 15	
10		
	GGC ATA AAG CCT ACT GCT GGA CAA GAC GTG GAA GAA CTC TTG ATC CCT	213
	Gly Ile Lys Pro Thr Ala Gly Gln Asp Val Glu Glu Leu Leu Ile Pro	
	20 25 30	
15		
	AAA GTT TGG GTG CCA CCT GAG GAT CCG CTT GCC AGC CCT AGT CGA CTG	261
	Lys Val Trp Val Pro Pro Glu Asp Pro Leu Ala Ser Pro Ser Arg Leu	
	35 40 45 50	
20		
	GCA AAG TTC CTC AGA GAG AAC GGC TAC AAA GTT TTG CAG CCA CGG TCT	309
	Ala Lys Phe Leu Arg Glu Asn Gly Tyr Lys Val Leu Gln Pro Arg Ser	
	55 60 65	
25		
	CTG CCC GAG AAT GAG GAG TAT GAG ACC GAC CAA ATA CTC CCA GAC TTA	357
	Leu Pro Glu Asn Glu Glu Tyr Glu Thr Asp Gln Ile Leu Pro Asp Leu	
	70 75 80	
30		
	GCA TGG ATG CGA CAG ATA GAA GGG GCT GTT TTA AAA CCC ACT CTA TCT	405
	Ala Trp Met Arg Gln Ile Glu Gly Ala Val Leu Lys Pro Thr Leu Ser	
	85 90 95	
35		
	CTC CCT ATT GGA GAT CAG GAG TAC TTC CCA AAG TAC TAC CCA ACA CAT	453
	Leu Pro Ile Gly Asp Gln Glu Tyr Phe Pro Lys Tyr Tyr Pro Thr His	
	100 105 110	
40		
	CGC CCT AGC AAG GAG AAG CCC AAT GCG TAC CCG CCA GAC ATC GCA CTA	501
	Arg Pro Ser Lys Glu Lys Pro Asn Ala Tyr Pro Pro Asp Ile Ala Leu	
	115 120 125 130	
45		
	CTC AAG CAG ATG ATT TAC CTG TTT CTC CAG GTT CCA GAG GCC AAC GAG	549
	Leu Lys Gln Met Ile Tyr Leu Phe Leu Gln Val Pro Glu Ala Asn Glu	
	135 140 145	
50		
	GGC CTA AAG GAT GAA GTA ACC CTC TTG ACC CAA AAC ATA AGG GAC AAG	597
	Gly Leu Lys Asp Glu Val Thr Leu Leu Thr Gln Asn Ile Arg Asp Lys	
	150 155 160	
55		
	GCC TAT GGA AGT GGG ACC TAC ATG GGA CAA GCA AAT CGA CTT GTG GCC	645
	Ala Tyr Gly Ser Gly Thr Tyr Met Gly Gln Ala Asn Arg Leu Val Ala	
	165 170 175	

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	ATG AAG GAG GTC GCC ACT GGA AGA AAC CCA AAC AAG GAT CCT CTA AAG	693
	Met Lys Glu Val Ala Thr Gly Arg Asn Pro Asn Lys Asp Pro Leu Lys	
	180 185 190	
5		
	CTT GGG TAC ACT TTT GAG AGC ATC GCG CAG CTA CTT GAC ATC ACA CTA	741
	Leu Gly Tyr Thr Phe Glu Ser Ile Ala Gln Leu Leu Asp Ile Thr Leu	
	195 200 205 210	
10		
	CCG GTA GGC CCA CCC GGT GAG GAT GAC AAG CCC TGG GTG CCA CTC ACA	789
	Pro Val Gly Pro Pro Gly Glu Asp Asp Lys Pro Trp Val Pro Leu Thr	
	215 220 225	
15		
	AGA GTG CCG TCA CGG ATG TTG GTG CTG ACG GGA GAC GTA GAT GGC GAC	837
	Arg Val Pro Ser Arg Met Leu Val Leu Thr Gly Asp Val Asp Gly Asp	
	230 235 240	
20		
	TTT GAG GTT GAA GAT TAC CTT CCC AAA ATC AAC CTC AAG TCA TCA AGT	885
	Phe Glu Val Glu Asp Tyr Leu Pro Lys Ile Asn Leu Lys Ser Ser Ser	
	245 250 255	
25		
	GGA CTA CCA TAT GTA GGT CGC ACC AAA GGA GAG ACA ATT GGC GAG ATG	933
	Gly Leu Pro Tyr Val Gly Arg Thr Lys Gly Glu Thr Ile Gly Glu Met	
	260 265 270	
30		
	ATA GCT ATC TCA AAC CAG TTT CTC AGA GAG CTA TCA ACA CTG TTG AAG	981
	Ile Ala Ile Ser Asn Gln Phe Leu Arg Glu Leu Ser Thr Leu Leu Lys	
	275 280 285 290	
35		
	CAA GGT GCA GGG ACA AAG GGG TCA AAC AAG AAG AAG CTA CTC AGC ATG	1029
	Gln Gly Ala Gly Thr Lys Gly Ser Asn Lys Lys Lys Leu Leu Ser Met	
	295 300 305	
40		
	TTA AGT GAC TAT TGG TAC TTA TCA TGC GGG CTT TTG TTT CCA AAG GCT	1077
	Leu Ser Asp Tyr Trp Tyr Leu Ser Cys Gly Leu Leu Phe Pro Lys Ala	
	310 315 320	
45		
	GAA AGG TAC GAC AAA AGT ACA TGG CTC ACC AAG ACC CGG AAC ATA TGG	1125
	Glu Arg Tyr Asp Lys Ser Thr Trp Leu Thr Lys Thr Arg Asn Ile Trp	
	325 330 335	
50		
	TCA GCT CCA TCC CCA ACA CAC CTC ATG ATC TCT ATG ATC ACC TGG CCC	1173
	Ser Ala Pro Ser Pro Thr His Leu Met Ile Ser Met Ile Thr Trp Pro	
	340 345 350	
55		
	GTG ATG TCC AAC AGC CCA AAT AAC GTG TTG AAC ATT GAA GGG TGT CCA	1221
	Val Met Ser Asn Ser Pro Asn Asn Val Leu Asn Ile Glu Gly Cys Pro	
	355 360 365 370	

EP 0 887 412 B1

	TCA CTC TAC AAA TTC AAC CCG TTC AGA GGA GGG TTG AAC AGG ATC GTC	1269
	Ser Leu Tyr Lys Phe Asn Pro Phe Arg Gly Gly Leu Asn Arg Ile Val	
	375 380 385	
5	GAG TGG ATA TTG GCC CCG GAA GAA CCC AAG GCT CTT GTA TAT GCG GAC	1317
	Glu Trp Ile Leu Ala Pro Glu Glu Pro Lys Ala Leu Val Tyr Ala Asp	
	390 395 400	
10	AAC ATA TAC ATT GTC CAC TCA AAC ACG TGG TAC TCA ATT GAC CTA GAG	1365
	Asn Ile Tyr Ile Val His Ser Asn Thr Trp Tyr Ser Ile Asp Leu Glu	
	405 410 415	
15	AAG GGT GAG GCA AAC TGC ACT CGC CAA CAC ATG CAA GCC GCA ATG TAC	1413
	Lys Gly Glu Ala Asn Cys Thr Arg Gln His Met Gln Ala Ala Met Tyr	
	420 425 430	
20	TAC ATA CTC ACC AGA GGG TGG TCA GAC AAC GGC GAC CCA ATG TTC AAT	1461
	Tyr Ile Leu Thr Arg Gly Trp Ser Asp Asn Gly Asp Pro Met Phe Asn	
	435 440 445 450	
25	CAA ACA TGG GCC ACC TTT GCC ATG AAC ATT GCC CCT GCT CTA GTG GTG	1509
	Gln Thr Trp Ala Thr Phe Ala Met Asn Ile Ala Pro Ala Leu Val Val	
	455 460 465	
30	GAC TCA TCG TGC CTG ATA ATG AAC CTG CAA ATT AAG ACC TAT GGT CAA	1557
	Asp Ser Ser Cys Leu Ile Met Asn Leu Gln Ile Lys Thr Tyr Gly Gln	
	470 475 480	
35	GGC AGC GGG AAT GCA GCC ACG TTC ATC AAC AAC CAC CTC TTG AGC ACA	1605
	Gly Ser Gly Asn Ala Ala Thr Phe Ile Asn Asn His Leu Leu Ser Thr	
	485 490 495	
40	CTA GTG CTT GAC CAG TGG AAC CTG ATG AGA CAG CCC AGA CCA GAC AGC	1653
	Leu Val Leu Asp Gln Trp Asn Leu Met Arg Gln Pro Arg Pro Asp Ser	
	500 505 510	
45	GAG GAG TTC AAA TCA ATT GAG GAC AAG CTA GGT ATC AAC TTT AAG ATT	1701
	Glu Glu Phe Lys Ser Ile Glu Asp Lys Leu Gly Ile Asn Phe Lys Ile	
	515 520 525 530	
50	GAG AGG TCC ATT GAT GAT ATC AGG GGC AAG CTG AGA CAG CTT GTC CTC	1749
	Glu Arg Ser Ile Asp Asp Ile Arg Gly Lys Leu Arg Gln Leu Val Leu	
	535 540 545	
55	CTT GCA CAA CCA GGG TAC CTG AGT GGG GGG GTT GAA CCA GAA CAA TCC	1797
	Leu Ala Gln Pro Gly Tyr Leu Ser Gly Gly Val Glu Pro Glu Gln Ser	
	550 555 560	

EP 0 887 412 B1

	AGC CCA ACT GTT GAG CTT GAC CTA CTA GGG TGG TCA GCT ACA TAC AGC	1845
	Ser Pro Thr Val Glu Leu Asp Leu Leu Gly Trp Ser Ala Thr Tyr Ser	
	565 570 575	
5		
	AAA GAT CTC GGG ATC TAT GTG CCG GTG CTT GAC AAG GAA CGC CTA TTT	1893
	Lys Asp Leu Gly Ile Tyr Val Pro Val Leu Asp Lys Glu Arg Leu Phe	
	580 585 590	
10		
	TGT TCT GCT GCG TAT CCC AAG GGA GTA GAG AAC AAG AGT CTC AAG TCC	1941
	Cys Ser Ala Ala Tyr Pro Lys Gly Val Glu Asn Lys Ser Leu Lys Ser	
	595 600 605 610	
15		
	AAA GTC GGG ATC GAG CAG GCA TAC AAG GTA GTC AGG TAT GAG GCG TTG	1989
	Lys Val Gly Ile Glu Gln Ala Tyr Lys Val Val Arg Tyr Glu Ala Leu	
	615 620 625	
20		
	AGG TTG GTA GGT GGT TGG AAC TAC CCA CTC CTG AAC AAA GCC TGC AAG	2037
	Arg Leu Val Gly Gly Trp Asn Tyr Pro Leu Leu Asn Lys Ala Cys Lys	
	630 635 640	
25		
	AAT AAC GCA GGC GCC GCT CGG CGG CAT CTG GAG GCC AAG GGG TTC CCA	2085
	Asn Asn Ala Gly Ala Ala Arg Arg His Leu Glu Ala Lys Gly Phe Pro	
	645 650 655	
30		
	CTC GAC GAG TTC CTA GCC GAG TGG TCT GAG CTG TCA GAG TTC GGT GAG	2133
	Leu Asp Glu Phe Leu Ala Glu Trp Ser Glu Leu Ser Glu Phe Gly Glu	
	660 665 670	
35		
	GCC TTC GAA GGC TTC AAT ATC AAG CTG ACC GTA ACA TCT GAG AGC CTA	2181
	Ala Phe Glu Gly Phe Asn Ile Lys Leu Thr Val Thr Ser Glu Ser Leu	
	675 680 685 690	
40		
	GCC GAA CTG AAC AAG CCA GTA CCC CCC AAG CCC CCA AAT GTC AAC AGA	2229
	Ala Glu Leu Asn Lys Pro Val Pro Pro Lys Pro Pro Asn Val Asn Arg	
	695 700 705	
45		
	CCA GTC AAC ACT GGG GGA CTC AAG GCA GTC AGC AAC GCC CTC AAG ACC	2277
	Pro Val Asn Thr Gly Gly Leu Lys Ala Val Ser Asn Ala Leu Lys Thr	
	710 715 720	
50		
	GGT CGG TAC AGG AAC GAA GCC GGA CTG AGT GGT CTC GTC CTT CTA GCC	2325
	Gly Arg Tyr Arg Asn Glu Ala Gly Leu Ser Gly Leu Val Leu Leu Ala	
	725 730 735	
55		
	ACA GCA AGA AGC CGT CTG CAA GAT GCA GTT AAG GCC AAG GCA GAA GCC	2373
	Thr Ala Arg Ser Arg Leu Gln Asp Ala Val Lys Ala Lys Ala Glu Ala	
	740 745 750	

5 GAG AAA CTC CAC AAG TCC AAG CCA GAC GAC CCC GAT GCA GAC TGG TTC 2421  
 Glu Lys Leu His Lys Ser Lys Pro Asp Asp Pro Asp Ala Asp Trp Phe  
 755 760 765 770

10 GAA AGA TCA GAA ACT CTG TCA GAC CTT CTG GAG AAA GCC GAC ATC GCC 2469  
 Glu Arg Ser Glu Thr Leu Ser Asp Leu Leu Glu Lys Ala Asp Ile Ala  
 775 780 785

15 AGC AAG GTC GCC CAC TCA GCA CTC GTG GAA ACA AGC GAC GCC CTT GAA 2517  
 Ser Lys Val Ala His Ser Ala Leu Val Glu Thr Ser Asp Ala Leu Glu  
 790 795 800

20 GCA GTT CAG TCG ACT TCC GTG TAC ACC CCC AAG TAC CCA GAA GTC AAG 2565  
 Ala Val Gln Ser Thr Ser Val Tyr Thr Pro Lys Tyr Pro Glu Val Lys  
 805 810 815

25 AAC CCA CAG ACC GCC TCC AAC CCC GTT GTT GGG CTC CAC CTG CCC GCC 2613  
 Asn Pro Gln Thr Ala Ser Asn Pro Val Val Gly Leu His Leu Pro Ala  
 820 825 830

30 AAG AGA GCC ACC GGT GTC CAG GCC GCT CTT CTC GGA GCA GGA ACG AGC 2661  
 Lys Arg Ala Thr Gly Val Gln Ala Ala Leu Leu Gly Ala Gly Thr Ser  
 835 840 845 850

35 AGA CCA ATG GGG ATG GAG GCC CCA ACA CGG TCC AAG AAC GCC GTG AAA 2709  
 Arg Pro Met Gly Met Glu Ala Pro Thr Arg Ser Lys Asn Ala Val Lys  
 855 860 865

40 ATG GCC AAA CGG CGG CAA CGC CAA AAG GAG AGC CGC TAACAGCCAT 2755  
 Met Ala Lys Arg Arg Gln Arg Gln Lys Glu Ser Arg  
 870 875

45 GATGGGAACC ACTCAAGAAG AGGACACTAA TCCCAGACCC CGTATCCCCG GCCTTCGCCT 2815  
 GCGGGGGCCC CC 2827

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 878 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

EP 0 887 412 B1

	Met Ser Asp Ile Phe Asn Ser Pro Gln Ala Arg Ser Thr Ile Ser Ala	
	1 5 10 15	
5	Ala Phe Gly Ile Lys Pro Thr Ala Gly Gln Asp Val Glu Glu Leu Leu	
	20 25 30	
10	Ile Pro Lys Val Trp Val Pro Pro Glu Asp Pro Leu Ala Ser Pro Ser	
	35 40 45	
	Arg Leu Ala Lys Phe Leu Arg Glu Asn Gly Tyr Lys Val Leu Gln Pro	
	50 55 60	
15	Arg Ser Leu Pro Glu Asn Glu Glu Tyr Glu Thr Asp Gln Ile Leu Pro	
	65 70 75 80	
20	Asp Leu Ala Trp Met Arg Gln Ile Glu Gly Ala Val Leu Lys Pro Thr	
	85 90 95	
	Leu Ser Leu Pro Ile Gly Asp Gln Glu Tyr Phe Pro Lys Tyr Tyr Pro	
	100 105 110	
25	Thr His Arg Pro Ser Lys Glu Lys Pro Asn Ala Tyr Pro Pro Asp Ile	
	115 120 125	
30	Ala Leu Leu Lys Gln Met Ile Tyr Leu Phe Leu Gln Val Pro Glu Ala	
	130 135 140	
	Asn Glu Gly Leu Lys Asp Glu Val Thr Leu Leu Thr Gln Asn Ile Arg	
35	145 150 155 160	
	Asp Lys Ala Tyr Gly Ser Gly Thr Tyr Met Gly Gln Ala Asn Arg Leu	
	165 170 175	
40	Val Ala Met Lys Glu Val Ala Thr Gly Arg Asn Pro Asn Lys Asp Pro	
	180 185 190	
45	Leu Lys Leu Gly Tyr Thr Phe Glu Ser Ile Ala Gln Leu Leu Asp Ile	
	195 200 205	
	Thr Leu Pro Val Gly Pro Pro Gly Glu Asp Asp Lys Pro Trp Val Pro	
	210 215 220	
50	Leu Thr Arg Val Pro Ser Arg Met Leu Val Leu Thr Gly Asp Val Asp	
	225 230 235 240	
55	Gly Asp Phe Glu Val Glu Asp Tyr Leu Pro Lys Ile Asn Leu Lys Ser	
	245 250 255	

## EP 0 887 412 B1

	Ser Ser Gly Leu Pro Tyr Val Gly Arg Thr Lys Gly Glu Thr Ile Gly	
	260	270
5	Glu Met Ile Ala Ile Ser Asn Gln Phe Leu Arg Glu Leu Ser Thr Leu	
	275	285
10	Leu Lys Gln Gly Ala Gly Thr Lys Gly Ser Asn Lys Lys Lys Leu Leu	
	290	300
	Ser Met Leu Ser Asp Tyr Trp Tyr Leu Ser Cys Gly Leu Leu Phe Pro	
	305	320
15	Lys Ala Glu Arg Tyr Asp Lys Ser Thr Trp Leu Thr Lys Thr Arg Asn	
	325	335
20	Ile Trp Ser Ala Pro Ser Pro Thr His Leu Met Ile Ser Met Ile Thr	
	340	350
	Trp Pro Val Met Ser Asn Ser Pro Asn Asn Val Leu Asn Ile Glu Gly	
	355	365
25	Cys Pro Ser Leu Tyr Lys Phe Asn Pro Phe Arg Gly Gly Leu Asn Arg	
	370	380
30	Ile Val Glu Trp Ile Leu Ala Pro Glu Glu Pro Lys Ala Leu Val Tyr	
	385	400
	Ala Asp Asn Ile Tyr Ile Val His Ser Asn Thr Trp Tyr Ser Ile Asp	
	405	415
35	Leu Glu Lys Gly Glu Ala Asn Cys Thr Arg Gln His Met Gln Ala Ala	
	420	430
40	Met Tyr Tyr Ile Leu Thr Arg Gly Trp Ser Asp Asn Gly Asp Pro Met	
	435	445
	Phe Asn Gln Thr Trp Ala Thr Phe Ala Met Asn Ile Ala Pro Ala Leu	
	450	460
45	Val Val Asp Ser Ser Cys Leu Ile Met Asn Leu Gln Ile Lys Thr Tyr	
	465	480
50	Gly Gln Gly Ser Gly Asn Ala Ala Thr Phe Ile Asn Asn His Leu Leu	
	485	495
55	Ser Thr Leu Val Leu Asp Gln Trp Asn Leu Met Arg Gln Pro Arg Pro	
	500	510

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	Asp Ser Glu Glu Phe Lys Ser Ile Glu Asp Lys Leu Gly Ile Asn Phe	
	515	520 525
5	Lys Ile Glu Arg Ser Ile Asp Asp Ile Arg Gly Lys Leu Arg Gln Leu	
	530	535 540
10	Val Leu Leu Ala Gln Pro Gly Tyr Leu Ser Gly Gly Val Glu Pro Glu	
	545	550 555 560
	Gln Ser Ser Pro Thr Val Glu Leu Asp Leu Leu Gly Trp Ser Ala Thr	
		565 570 575
15	Tyr Ser Lys Asp Leu Gly Ile Tyr Val Pro Val Leu Asp Lys Glu Arg	
	580	585 590
20	Leu Phe Cys Ser Ala Ala Tyr Pro Lys Gly Val Glu Asn Lys Ser Leu	
	595	600 605
	Lys Ser Lys Val Gly Ile Glu Gln Ala Tyr Lys Val Val Arg Tyr Glu	
	610	615 620
25	Ala Leu Arg Leu Val Gly Gly Trp Asn Tyr Pro Leu Leu Asn Lys Ala	
	625	630 635 640
30	Cys Lys Asn Asn Ala Gly Ala Ala Arg Arg His Leu Glu Ala Lys Gly	
		645 650 655
	Phe Pro Leu Asp Glu Phe Leu Ala Glu Trp Ser Glu Leu Ser Glu Phe	
35		660 665 670
	Gly Glu Ala Phe Glu Gly Phe Asn Ile Lys Leu Thr Val Thr Ser Glu	
	675	680 685
40	Ser Leu Ala Glu Leu Asn Lys Pro Val Pro Pro Lys Pro Pro Asn Val	
	690	695 700
	Asn Arg Pro Val Asn Thr Gly Gly Leu Lys Ala Val Ser Asn Ala Leu	
45	705	710 715 720
	Lys Thr Gly Arg Tyr Arg Asn Glu Ala Gly Leu Ser Gly Leu Val Leu	
		725 730 735
50	Leu Ala Thr Ala Arg Ser Arg Leu Gln Asp Ala Val Lys Ala Lys Ala	
	740	745 750
	Glu Ala Glu Lys Leu His Lys Ser Lys Pro Asp Asp Pro Asp Ala Asp	
55	755	760 765



Trp Phe Glu Arg Ser Glu Thr Leu Ser Asp Leu Leu Glu Lys Ala Asp  
 770 775 780  
 5  
 Ile Ala Ser Lys Val Ala His Ser Ala Leu Val Glu Thr Ser Asp Ala  
 785 790 795 800  
 10  
 Leu Glu Ala Val Gln Ser Thr Ser Val Tyr Thr Pro Lys Tyr Pro Glu  
 805 810 815  
 Val Lys Asn Pro Gln Thr Ala Ser Asn Pro Val Val Gly Leu His Leu  
 15 820 825 830  
 Pro Ala Lys Arg Ala Thr Gly Val Gln Ala Ala Leu Leu Gly Ala Gly  
 835 840 845  
 20  
 Thr Ser Arg Pro Met Gly Met Glu Ala Pro Thr Arg Ser Lys Asn Ala  
 850 855 860  
 Val Lys Met Ala Lys Arg Arg Gln Arg Gln Lys Glu Ser Arg  
 25 865 870 875

30 (2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

35 (A) LENGTH: 3261 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

45 (A) NAME/KEY: CDS  
 (B) LOCATION: 97..531

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

50 GGATACGATC GGTCTGACCC CGGGGGAGTC ACCCGGGGAC AGGCCGTCAA GGCCTTGTTT 60  
 CAGGATGGGA CTCCTCCTTC TACAACGCTA TCATTG ATG GTT AGT AGA GAT CAG 114  
 Met Val Ser Arg Asp Gln  
 1 5

55

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5	ACA AAC GAT CGC AGC GAT GAC AAA CCT GCA AGA TCA AAC CCA ACA GAT Thr Asn Asp Arg Ser Asp Asp Lys Pro Ala Arg Ser Asn Pro Thr Asp 10 15 20	162
10	TGT TCC GTT CAT ACG GAG CCT TCT GAT GCC AAC AAC CGG ACC GGC GTC Cys Ser Val His Thr Glu Pro Ser Asp Ala Asn Asn Arg Thr Gly Val 25 30 35	210
15	CAT TCC GGA CGA CAC CCT GGA GAA GCA CAC TCT CAG GTC AGA GAC CTC His Ser Gly Arg His Pro Gly Glu Ala His Ser Gln Val Arg Asp Leu 40 45 50	258
20	GAC CTA CAA TTT GAC TGT GGG GGA CAC AGG GTC AGG GCT AAT TGT CTT Asp Leu Gln Phe Asp Cys Gly Gly His Arg Val Arg Ala Asn Cys Leu 55 60 65 70	306
25	TTT CCC TGG ATT CCC TGG CTC AAT TGT GGG TGC TCA CTA CAC ACT GCA Phe Pro Trp Ile Pro Trp Leu Asn Cys Gly Cys Ser Leu His Thr Ala 75 80 85	354
30	GGG CAA TGG GAA CTA CAA GTT CGA TCA GAT GCT CCT GAC TGC CCA GAA Gly Gln Trp Glu Leu Gln Val Arg Ser Asp Ala Pro Asp Cys Pro Glu 90 95 100	402
35	CCT ACC GGC CAG TTA CAA CTA CTG CAG GCT AGT GAG TCG GAG TCT CAC Pro Thr Gly Gln Leu Gln Leu Leu Gln Ala Ser Glu Ser Glu Ser His 105 110 115	450
40	AGT GAG GTC AAG CAC ACT TCC TGG TGG CGT TTG TGC ACT AAA CGG CAC Ser Glu Val Lys His Thr Ser Trp Trp Arg Leu Cys Thr Lys Arg His 120 125 130	498
45	CAT AAA CGC CGT GAC CTT CCA AGG AAG CCT GAG TGA ACT GACA GAT GTTAGCT His Lys Arg Arg Asp Leu Pro Arg Lys Pro Glu 135 140 145	551
50	ACAATGGGTT GATGTCTGCA ACAGCCAACA TCAACGACAA AATTGGGAAC GTCCTAGTAG GGGAAGGGGT CACCGTCCTC AGCTTACCCA CATCATATGA TCTTGGGTAT GTGAGGCTTG GTGACCCCAT TCCCGCAATA GGGCTTGACC CAAAAATGGT AGCCACATGT GACAGCAGTG ACAGGCCCAG AGTCTACACC ATA ACTGCAG CCGATGATTA CCAATTCTCA TCACAGTACC AACCAGGTGG GGTAACAATC AACTGTTCT CAGCCAACAT TGATGCCATC ACAAGCCTCA GCGTTGGGGG AGAGCTCGTG TTTCAAACAA GCGTCCACGG CCTTG TACTG GGC GCCACCA	611 671 731 791 851 911

EP 0 887 412 B1

	TCTACCTCAT AGGCTTTGAT GGGACAACGG TAATCACCAG GGCTGTGGCC GCAAACAATG	971
5	GGCTGACGAC CGGCACCGAC AACCTTATGC CATTCAATCT TGTGATTCCA ACAAACGAGA	1031
	TAACCCAGCC AATCACATCC ATCAAACCTGG AGATAGTGAC CTCCAAAAGT GGTGGTCAGG	1091
10	CAGGGGATCA GATGTCATGG TCGGCAAGAG GGAGCCTAGC AGTGACGATC CATGGTGGCA	1151
	ACTATCCAGG GGCCCTCCGT CCCGTCACGC TAGTGGCCTA CGAAAGAGTG GCAACAGGAT	1211
15	CCGTCGTTAC GGTGCTGGG GTGAGCAACT TCGAGCTGAT CCCAAATCCT GAACTAGCAA	1271
	AGAACCTGGT TACAGAATAC GGCCGATTTG ACCCAGGAGC CATGAACTAC ACAAATTTGA	1331
20	TACTGAGTGA GAGGGACCGT CTTGGCATCA AGACCGTCTG GCCAACAAGG GAGTACACTG	1391
	ACTTTCGTGA ATACTTCATG GAGGTGGCCG ACCTCAACTC TCCCCTGAAG ATTGCAGGAG	1451
25	CATTGCGCTT CAAAGACATA ATCCGGGCCA TAAGGAGGAT AGCTGTGCCG GTGGTCTCCA	1511
	CATTGTTCCC ACCTGCCGCT CCCCTAGCCC ATGCAATTGG GGAAGGTGTA GACTACCTGC	1571
30	TGGGCGATGA GGCACAGGCT GCTTCAGGAA CTGCTCGAGC CGCGTCAGGA AAAGCAAGAG	1631
	CTGCCTCAGG CCGCATAAGG CAGCTGACTC TCGCCGCCGA CAAGGGGTAC GAGGTAGTCG	1691
35	CGAATCTATT CCAGGTGCCC CAGAATCCCG TAGTCGACGG GATTCTTGCT TCACCTGGGG	1751
	TACTCCGCGG TGCACACAAC CTCGACTGCG TGTTAAGAGA GGGTGCCACG CTATTCCCTG	1811
40	TGGTTATTAC GACAGTGGA GACGCCATGA CACCCAAAGC ATTGAACAGC AAAATGTTTG	1871
	CTGTCATTGA AGGCGTGCGA GAAGACCTCC AACCTCCATC TCAAAGAGGA TCCTTCATAC	1931
45	GAACTCTCTC TGGACACAGA GTCTATGGAT ATGCTCCAGA TGGGGTACTT CCACTGGAGA	1991
	CTGGGAGAGA CTACACCGTT GTCCCAATAG ATGATGTCTG GGACGACAGC ATTATGCTGT	2051
50	CCAAAGATCC CATACTCCT ATTGTGGGAA ACAGTGGAAT TCTAGCCATA GCTTACATGG	2111
	ATGTGTTTCG ACCCAAAGTC CCAATCCATG TGGCTATGAC GGGAGCCCTC AATGCTTGTG	2171
55	GCGAGATTGA GAAAGTAAGC TTAGAAGCA CCAAGCTCGC CACTGCACAC CGACTTGGCC	2231
	TTAGGTTGGC TGGTCCCGGA GCATTGATG TAAACACCGG GCCCAACTGG GCAACGTTCA	2291
	TCAAACGTTT CCCTCACAAT CCACGCGACT GGGACAGGCT CCCCTACCTC AACCTACCAT	2351

ACCTTCCACC CAATGCAGGA CGCCAGTACC ACCTTGCCAT GGCTGCATCA GAGTTCAAAG 2411  
 5 AGACCCCCGA ACTCGAGAGT GCCGTCAGAG CAATGGAAGC AGCAGCCAAC GTGGACCCAC 2471  
 TATTCCAATC TGCACTCAGT GTGTTCATGT GGCTGGAAGA GAATGGGATT GTGACTGACA 2531  
 10 TGGCCAACTT CGCACTCAGC GACCCGAACG CCCATCGGAT GCGAAATTTT CTTGCAAACG 2591  
 CACCACAAGC AGGCAGCAAG TCGCAAAGGG CCAAGTACGG GACAGCAGGC TACGGAGTGG 2651  
 15 AGGCTCGGGG CCCACACCA GAGGAAGCAC AGAGGGAAAA AGACACACGG ATCTCAAAGA 2711  
 AGATGGAGAC CATGGGCATC TACTTTGCAA CACCAGAATG GGTAGCACTC AATGGGCACC 2771  
 GAGGGCCAAG CCCCGGCCAG CTAAAGTACT GGCAGAACAC ACGAGAAATA CCGGACCCAA 2831  
 20 ACGAGGACTA TCTAGACTAC GTGCATGCAG AGAAGAGCCG GTTGGCATCA GAAGAACAAA 2891  
 TCCTAAGGGC AGCTACGTCG ATCTACGGGG CTCCAGGACA GGCAGAGCCA CCCCAAGCTT 2951  
 25 TCATAGACGA AGTTGCCAAA GTCTATGAAA TCAACCATGG ACGTGGCCCA AACCAAGAAC 3011  
 AGATGAAAGA TCTGCTCTTG ACTGCGATGG AGATGAAGCA TCGCAATCCC AGGCGGGGCTC 3071  
 30 TACCAAAGCC CAAGCCAAAA CCCAATGCTC CAACACAGAG ACCCCCTGGT CGGCTGGGCC 3131  
 GCTGGATCAG GACCGTCTCT GATGAGGACC TTGAGTGAGG CTCCTGGGAG TCTCCCGACA 3191  
 35 CCACCCGCGC AGGTGTGGAC ACCAATTCGG CCTTACAACA TCCCAAATTG GATCCGTTCC 3251  
 CGGGTCCCCT 3261

## (2) INFORMATION FOR SEQ ID NO: 4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 145 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met Val Ser Arg Asp Gln Thr Asn Asp Arg Ser Asp Asp Lys Pro Ala

55 1 5 10 15

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	CAGGATGGGA CTCCTCCTTC TACAACGCTA TCATTGATGG TTAGTAGAGA TCAGACAAAC	120
5	GATCGCAGCG ATG ACA AAC CTG CAA GAT CAA ACC CAA CAG ATT GTT CCG Met Thr Asn Leu Gln Asp Gln Thr Gln Gln Ile Val Pro 1 5 10	169
10	TTC ATA CGG AGC CTT CTG ATG CCA ACA ACC GGA CCG GCG TCC ATT CCG Phe Ile Arg Ser Leu Leu Met Pro Thr Thr Gly Pro Ala Ser Ile Pro 15 20 25	217
15	GAC GAC ACC CTG GAG AAG CAC ACT CTC AGG TCA GAG ACC TCG ACC TAC Asp Asp Thr Leu Glu Lys His Thr Leu Arg Ser Glu Thr Ser Thr Tyr 30 35 40 45	265
20	AAT TTG ACT GTG GGG GAC ACA GGG TCA GGG CTA ATT GTC TTT TTC CCT Asn Leu Thr Val Gly Asp Thr Gly Ser Gly Leu Ile Val Phe Phe Pro 50 55 60	313
25	GGA TTC CCT GGC TCA ATT GTG GGT GCT CAC TAC ACA CTG CAG GGC AAT Gly Phe Pro Gly Ser Ile Val Gly Ala His Tyr Thr Leu Gln Gly Asn 65 70 75	361
30	GGG AAC TAC AAG TTC GAT CAG ATG CTC CTG ACT GCC CAG AAC CTA CCG Gly Asn Tyr Lys Phe Asp Gln Met Leu Leu Thr Ala Gln Asn Leu Pro 80 85 90	409
35	GCC AGT TAC AAC TAC TGC AGG CTA GTG AGT CGG AGT CTC ACA GTG AGG Ala Ser Tyr Asn Tyr Cys Arg Leu Val Ser Arg Ser Leu Thr Val Arg 95 100 105	457
40	TCA AGC ACA CTT CCT GGT GGC GTT TAT GCA CTA AAC GGC ACC ATA AAC Ser Ser Thr Leu Pro Gly Gly Val Tyr Ala Leu Asn Gly Thr Ile Asn 110 115 120 125	505
45	GCC GTG ACC TTC CAA GGA AGC CTG AGT GAA CTG ACA GAT GTT AGC TAC Ala Val Thr Phe Gln Gly Ser Leu Ser Glu Leu Thr Asp Val Ser Tyr 130 135 140	553
50	AAT GGG TTG ATG TCT GCA ACA GCC AAC ATC AAC GAC AAA ATT GGG AAC Asn Gly Leu Met Ser Ala Thr Ala Asn Ile Asn Asp Lys Ile Gly Asn 145 150 155	601
55	GTC CTA GTA GGG GAA GGG GTC ACC GTC CTC AGC TTA CCC ACA TCA TAT Val Leu Val Gly Glu Gly Val Thr Val Leu Ser Leu Pro Thr Ser Tyr 160 165 170	649
	GAT CTT GGG TAT GTG AGG CTT GGT GAC CCC ATT CCC GCA ATA GGG CTT	697

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	Asp Leu Gly Tyr Val Arg Leu Gly Asp Pro Ile Pro Ala Ile Gly Leu	
	175 180 185	
5	GAC CCA AAA ATG GTA GCC ACA TGT GAC AGC AGT GAC AGG CCC AGA GTC	745
	Asp Pro Lys Met Val Ala Thr Cys Asp Ser Ser Asp Arg Pro Arg Val	
	190 195 200 205	
10	TAC ACC ATA ACT GCA GCC GAT GAT TAC CAA TTC TCA TCA CAG TAC CAA	793
	Tyr Thr Ile Thr Ala Ala Asp Asp Tyr Gln Phe Ser Ser Gln Tyr Gln	
	210 215 220	
15	CCA GGT GGG GTA ACA ATC ACA CTG TTC TCA GCC AAC ATT GAT GCC ATC	841
	Pro Gly Gly Val Thr Ile Thr Leu Phe Ser Ala Asn Ile Asp Ala Ile	
	225 230 235	
20	ACA AGC CTC AGC GTT GGG GGA GAG CTC GTG TTT CAA ACA AGC GTC CAC	889
	Thr Ser Leu Ser Val Gly Gly Glu Leu Val Phe Gln Thr Ser Val His	
	240 245 250	
25	GGC CTT GTA CTG GGC GCC ACC ATC TAC CTC ATA GGC TTT GAT GGG ACA	937
	Gly Leu Val Leu Gly Ala Thr Ile Tyr Leu Ile Gly Phe Asp Gly Thr	
	255 260 265	
30	ACG GTA ATC ACC AGG GCT GTG GCC GCA AAC AAT GGG CTG ACG ACC GGC	985
	Thr Val Ile Thr Arg Ala Val Ala Ala Asn Asn Gly Leu Thr Thr Gly	
	270 275 280 285	
35	ACC GAC AAC CTT ATG CCA TTC AAT CTT GTG ATT CCA ACA AAC GAG ATA	1033
	Thr Asp Asn Leu Met Pro Phe Asn Leu Val Ile Pro Thr Asn Glu Ile	
	290 295 300	
40	ACC CAG CCA ATC ACA TCC ATC AAA CTG GAG ATA GTG ACC TCC AAA AGT	1081
	Thr Gln Pro Ile Thr Ser Ile Lys Leu Glu Ile Val Thr Ser Lys Ser	
	305 310 315	
45	GGT GGT CAG GCA GGG GAT CAG ATG TCA TGG TCG GCA AGA GGG AGC CTA	1129
	Gly Gly Gln Ala Gly Asp Gln Met Ser Trp Ser Ala Arg Gly Ser Leu	
	320 325 330	
50	GCA GTG ACG ATC CAT GGT GGC AAC TAT CCA GGG GCC CTC CGT CCC GTC	1177
	Ala Val Thr Ile His Gly Gly Asn Tyr Pro Gly Ala Leu Arg Pro Val	
	335 340 345	
55	ACG CTA GTG GCC TAC GAA AGA GTG GCA ACA GGA TCC GTC GTT ACG GTC	1225
	Thr Leu Val Ala Tyr Glu Arg Val Ala Thr Gly Ser Val Val Thr Val	
	350 355 360 365	
55	GCT GGG GTG AGC AAC TTC GAG CTG ATC CCA AAT CCT GAA CTA GCA AAG	1273

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	Ala Gly Val Ser Asn Phe Glu Leu Ile Pro Asn Pro Glu Leu Ala Lys	
	370 375 380	
5	AAC CTG GTT ACA GAA TAC GGC CGA TTT GAC CCA GGA GCC ATG AAC TAC Asn Leu Val Thr Glu Tyr Gly Arg Phe Asp Pro Gly Ala Met Asn Tyr	1321
	385 390 395	
10	ACA AAA TTG ATA CTG AGT GAG AGG GAC CGT CTT GGC ATC AAG ACC GTC Thr Lys Leu Ile Leu Ser Glu Arg Asp Arg Leu Gly Ile Lys Thr Val	1369
	400 405 410	
15	TGG CCA ACA AGG GAG TAC ACT GAC TTT CGT GAA TAC TTC ATG GAG GTG Trp Pro Thr Arg Glu Tyr Thr Asp Phe Arg Glu Tyr Phe Met Glu Val	1417
	415 420 425	
20	GCC GAC CTC AAC TCT CCC CTG AAG ATT GCA GGA GCA TTC GGC TTC AAA Ala Asp Leu Asn Ser Pro Leu Lys Ile Ala Gly Ala Phe Gly Phe Lys	1465
	430 435 440 445	
25	GAC ATA ATC CGG GCC ATA AGG AGG ATA GCT GTG CCG GTG GTC TCC ACA Asp Ile Ile Arg Ala Ile Arg Arg Ile Ala Val Pro Val Val Ser Thr	1513
	450 455 460	
30	TTG TTC CCA CCT GCC GCT CCC CTA GCC CAT GCA ATT GGG GAA GGT GTA Leu Phe Pro Pro Ala Ala Pro Leu Ala His Ala Ile Gly Glu Gly Val	1561
	465 470 475	
35	GAC TAC CTG CTG GGC GAT GAG GCA CAG GCT GCT TCA GGA ACT GCT CGA Asp Tyr Leu Leu Gly Asp Glu Ala Gln Ala Ala Ser Gly Thr Ala Arg	1609
	480 485 490	
40	GCC GCG TCA GGA AAA GCA AGA GCT GCC TCA GGC CGC ATA AGG CAG CTG Ala Ala Ser Gly Lys Ala Arg Ala Ala Ser Gly Arg Ile Arg Gln Leu	1657
	495 500 505	
45	ACT CTC GCC GCC GAC AAG GGG TAC GAG GTA GTC GCG AAT CTA TTC CAG Thr Leu Ala Ala Asp Lys Gly Tyr Glu Val Val Ala Asn Leu Phe Gln	1705
	510 515 520 525	
50	GTG CCC CAG AAT CCC GTA GTC GAC GGG ATT CTT GCT TCA CCT GGG GTA Val Pro Gln Asn Pro Val Val Asp Gly Ile Leu Ala Ser Pro Gly Val	1753
	530 535 540	
55	CTC CGC GGT GCA CAC AAC CTC GAC TGC GTG TTA AGA GAG GGT GCC ACG Leu Arg Gly Ala His Asn Leu Asp Cys Val Leu Arg Glu Gly Ala Thr	1801
	545 550 555	
55	CTA TTC CCT GTG GTT ATT ACG ACA GTG GAA GAC GCC ATG ACA CCC AAA	1849



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	Leu Phe Pro Val Val Ile Thr Thr Val Glu Asp Ala Met Thr Pro Lys	
	560 565 570	
5	GCA TTG AAC AGC AAA ATG TTT GCT GTC ATT GAA GGC GTG CGA GAA GAC Ala Leu Asn Ser Lys Met Phe Ala Val Ile Glu Gly Val Arg Glu Asp	1897
	575 580 585	
10	CTC CAA CCT CCA TCT CAA AGA GGA TCC TTC ATA CGA ACT CTC TCT GGA Leu Gln Pro Pro Ser Gln Arg Gly Ser Phe Ile Arg Thr Leu Ser Gly	1945
	590 595 600 605	
15	CAC AGA GTC TAT GGA TAT GCT CCA GAT GGG GTA CTT CCA CTG GAG ACT His Arg Val Tyr Gly Tyr Ala Pro Asp Gly Val Leu Pro Leu Glu Thr	1993
	610 615 620	
20	GGG AGA GAC TAC ACC GTT GTC CCA ATA GAT GAT GTC TGG GAC GAC AGC Gly Arg Asp Tyr Thr Val Val Pro Ile Asp Asp Val Trp Asp Asp Ser	2041
	625 630 635	
25	ATT ATG CTG TCC AAA GAT CCC ATA CCT CCT ATT GTG GGA AAC AGT GGA Ile Met Leu Ser Lys Asp Pro Ile Pro Pro Ile Val Gly Asn Ser Gly	2089
	640 645 650	
30	AAT CTA GCC ATA GCT TAC ATG GAT GTG TTT CGA CCC AAA GTC CCA ATC Asn Leu Ala Ile Ala Tyr Met Asp Val Phe Arg Pro Lys Val Pro Ile	2137
	655 660 665	
35	CAT GTG GCT ATG ACG GGA GCC CTC AAT GCT TGT GGC GAG ATT GAG AAA His Val Ala Met Thr Gly Ala Leu Asn Ala Cys Gly Glu Ile Glu Lys	2185
	670 675 680 685	
40	GTA AGC TTT AGA AGC ACC AAG CTC GCC ACT GCA CAC CGA CTT GGC CTT Val Ser Phe Arg Ser Thr Lys Leu Ala Thr Ala His Arg Leu Gly Leu	2233
	690 695 700	
45	AGG TTG GCT GGT CCC GGA GCA TTC GAT GTA AAC ACC GGC CCC AAC TGG Arg Leu Ala Gly Pro Gly Ala Phe Asp Val Asn Thr Gly Pro Asn Trp	2281
	705 710 715	
50	GCA ACG TTC ATC AAA CGT TTC CCT CAC AAT CCA CGC GAC TGG GAC AGG Ala Thr Phe Ile Lys Arg Phe Pro His Asn Pro Arg Asp Trp Asp Arg	2329
	720 725 730	
55	CTC CCC TAC CTC AAC CTA CCA TAC CTT CCA CCC AAT GCA GGA CGC CAG Leu Pro Tyr Leu Asn Leu Pro Tyr Leu Pro Pro Asn Ala Gly Arg Gln	2377
	735 740 745	
55	TAC CAC CTT GCC ATG GCT GCA TCA GAG TTC AAA GAG ACC CCC GAA CTC	2425

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	Tyr	His	Leu	Ala	Met	Ala	Ala	Ser	Glu	Phe	Lys	Glu	Thr	Pro	Glu	Leu	
	750					755					760					765	
5	GAG	AGT	GCC	GTC	AGA	GCA	ATG	GAA	GCA	GCA	GCC	AAC	GTG	GAC	CCA	CTA	2473
	Glu	Ser	Ala	Val	Arg	Ala	Met	Glu	Ala	Ala	Ala	Asn	Val	Asp	Pro	Leu	
				770					775						780		
10	TTC	CAA	TCT	GCA	CTC	AGT	GTG	TTC	ATG	TGG	CTG	GAA	GAG	AAT	GGG	ATT	2521
	Phe	Gln	Ser	Ala	Leu	Ser	Val	Phe	Met	Trp	Leu	Glu	Glu	Asn	Gly	Ile	
				785					790						795		
15	GTG	ACT	GAC	ATG	GCC	AAC	TTC	GCA	CTC	AGC	GAC	CCG	AAC	GCC	CAT	CGG	2569
	Val	Thr	Asp	Met	Ala	Asn	Phe	Ala	Leu	Ser	Asp	Pro	Asn	Ala	His	Arg	
			800					805						810			
20	ATG	CGA	AAT	TTT	CTT	GCA	AAC	GCA	CCA	CAA	GCA	GGC	AGC	AAG	TCG	CAA	2617
	Met	Arg	Asn	Phe	Leu	Ala	Asn	Ala	Pro	Gln	Ala	Gly	Ser	Lys	Ser	Gln	
		815					820					825					
25	AGG	GCC	AAG	TAC	GGG	ACA	GCA	GGC	TAC	GGA	GTG	GAG	GCT	CGG	GGC	CCC	2665
	Arg	Ala	Lys	Tyr	Gly	Thr	Ala	Gly	Tyr	Gly	Val	Glu	Ala	Arg	Gly	Pro	
	830				835					840					845		
30	ACA	CCA	GAG	GAA	GCA	CAG	AGG	GAA	AAA	GAC	ACA	CGG	ATC	TCA	AAG	AAG	2713
	Thr	Pro	Glu	Glu	Ala	Gln	Arg	Glu	Lys	Asp	Thr	Arg	Ile	Ser	Lys	Lys	
				850					855						860		
35	ATG	GAG	ACC	ATG	GGC	ATC	TAC	TTT	GCA	ACA	CCA	GAA	TGG	GTA	GCA	CTC	2761
	Met	Glu	Thr	Met	Gly	Ile	Tyr	Phe	Ala	Thr	Pro	Glu	Trp	Val	Ala	Leu	
			865					870						875			
40	AAT	GGG	CAC	CGA	GGG	CCA	AGC	CCC	GGC	CAG	CTA	AAG	TAC	TGG	CAG	AAC	2809
	Asn	Gly	His	Arg	Gly	Pro	Ser	Pro	Gly	Gln	Leu	Lys	Tyr	Trp	Gln	Asn	
		880						885						890			
45	ACA	CGA	GAA	ATA	CCG	GAC	CCA	AAC	GAG	GAC	TAT	CTA	GAC	TAC	GTG	CAT	2857
	Thr	Arg	Glu	Ile	Pro	Asp	Pro	Asn	Glu	Asp	Tyr	Leu	Asp	Tyr	Val	His	
		895					900					905					
50	GCA	GAG	AAG	AGC	CGG	TTG	GCA	TCA	GAA	GAA	CAA	ATC	CTA	AGG	GCA	GCT	2905
	Ala	Glu	Lys	Ser	Arg	Leu	Ala	Ser	Glu	Glu	Gln	Ile	Leu	Arg	Ala	Ala	
	910				915						920				925		
55	ACG	TCG	ATC	TAC	GGG	GCT	CCA	GGA	CAG	GCA	GAG	CCA	CCC	CAA	GCT	TTC	2953
	Thr	Ser	Ile	Tyr	Gly	Ala	Pro	Gly	Gln	Ala	Glu	Pro	Pro	Gln	Ala	Phe	
				930					935						940		
60	ATA	GAC	GAA	GTT	GCC	AAA	GTC	TAT	GAA	ATC	AAC	CAT	GGA	CGT	GGC	CCA	3001

Ile Asp Glu Val Ala Lys Val Tyr Glu Ile Asn His Gly Arg Gly Pro  
                   945                  950                  955

5 AAC CAA GAA CAG ATG AAA GAT CTG CTC TTG ACT GCG ATG GAG ATG AAG 3049  
 Asn Gln Glu Gln Met Lys Asp Leu Leu Leu Thr Ala Met Glu Met Lys  
                   960                  965                  970

10 CAT CGC AAT CCC AGG CGG GCT CTA CCA AAG CCC AAG CCA AAA CCC AAT 3097  
 His Arg Asn Pro Arg Arg Ala Leu Pro Lys Pro Lys Pro Lys Pro Asn  
                   975                  980                  985

15 GCT CCA ACA CAG AGA CCC CCT GGT CGG CTG GGC CGC TGG ATC AGG ACC 3145  
 Ala Pro Thr Gln Arg Pro Pro Gly Arg Leu Gly Arg Trp Ile Arg Thr  
                   990                  995                  1000                  1005

20 GTC TCT GAT GAG GAC CTT GAG TGAGGCTCCT GGGAGTCTCC CGACACCACC 3196  
 Val Ser Asp Glu Asp Leu Glu  
                   1010

25 CGCGCAGGTG TGGACACCAA TTCGGCCTTA CAACATCCCA AATTGGATCC GTTCGCGGGT 3256  
 CCCCT 3261

(2) INFORMATION FOR SEQ ID NO: 6:

30 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1012 amino acids  
 (B) TYPE: amino acid  
 35 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

40 Met Thr Asn Leu Gln Asp Gln Thr Gln Gln Ile Val Pro Phe Ile Arg  
           1                  5                  10                  15

45 Ser Leu Leu Met Pro Thr Thr Gly Pro Ala Ser Ile Pro Asp Asp Thr  
                   20                  25                  30

50 Leu Glu Lys His Thr Leu Arg Ser Glu Thr Ser Thr Tyr Asn Leu Thr  
                   35                  40                  45

Val Gly Asp Thr Gly Ser Gly Leu Ile Val Phe Phe Pro Gly Phe Pro  
           50                  55                  60

55 Gly Ser Ile Val Gly Ala His Tyr Thr Leu Gln Gly Asn Gly Asn Tyr

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	65	70	75	80
5	Lys Phe Asp Gln Met Leu Leu Thr Ala Gln Asn Leu Pro Ala Ser Tyr	85	90	95
10	Asn Tyr Cys Arg Leu Val Ser Arg Ser Leu Thr Val Arg Ser Ser Thr	100	105	110
15	Leu Pro Gly Gly Val Tyr Ala Leu Asn Gly Thr Ile Asn Ala Val Thr	115	120	125
20	Phe Gln Gly Ser Leu Ser Glu Leu Thr Asp Val Ser Tyr Asn Gly Leu	130	135	140
25	Met Ser Ala Thr Ala Asn Ile Asn Asp Lys Ile Gly Asn Val Leu Val	145	150	155
30	Gly Glu Gly Val Thr Val Leu Ser Leu Pro Thr Ser Tyr Asp Leu Gly	165	170	175
35	Tyr Val Arg Leu Gly Asp Pro Ile Pro Ala Ile Gly Leu Asp Pro Lys	180	185	190
40	Met Val Ala Thr Cys Asp Ser Ser Asp Arg Pro Arg Val Tyr Thr Ile	195	200	205
45	Thr Ala Ala Asp Asp Tyr Gln Phe Ser Ser Gln Tyr Gln Pro Gly Gly	210	215	220
50	Val Thr Ile Thr Leu Phe Ser Ala Asn Ile Asp Ala Ile Thr Ser Leu	225	230	235
55	Ser Val Gly Gly Glu Leu Val Phe Gln Thr Ser Val His Gly Leu Val	245	250	255
60	Leu Gly Ala Thr Ile Tyr Leu Ile Gly Phe Asp Gly Thr Thr Val Ile	260	265	270
65	Thr Arg Ala Val Ala Ala Asn Asn Gly Leu Thr Thr Gly Thr Asp Asn	275	280	285
70	Leu Met Pro Phe Asn Leu Val Ile Pro Thr Asn Glu Ile Thr Gln Pro	290	295	300
75	Ile Thr Ser Ile Lys Leu Glu Ile Val Thr Ser Lys Ser Gly Gly Gln	305	310	315
80	Ala Gly Asp Gln Met Ser Trp Ser Ala Arg Gly Ser Leu Ala Val Thr			320

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	325	330	335
5	Ile His Gly Gly Asn Tyr Pro Gly Ala Leu Arg Pro Val Thr Leu Val 340 345 350		
10	Ala Tyr Glu Arg Val Ala Thr Gly Ser Val Val Thr Val Ala Gly Val 355 360 365		
15	Ser Asn Phe Glu Leu Ile Pro Asn Pro Glu Leu Ala Lys Asn Leu Val 370 375 380		
20	Thr Glu Tyr Gly Arg Phe Asp Pro Gly Ala Met Asn Tyr Thr Lys Leu 385 390 395 400		
25	Ile Leu Ser Glu Arg Asp Arg Leu Gly Ile Lys Thr Val Trp Pro Thr 405 410 415		
30	Arg Glu Tyr Thr Asp Phe Arg Glu Tyr Phe Met Glu Val Ala Asp Leu 420 425 430		
35	Asn Ser Pro Leu Lys Ile Ala Gly Ala Phe Gly Phe Lys Asp Ile Ile 435 440 445		
40	Arg Ala Ile Arg Arg Ile Ala Val Pro Val Val Ser Thr Leu Phe Pro 450 455 460		
45	Pro Ala Ala Pro Leu Ala His Ala Ile Gly Glu Gly Val Asp Tyr Leu 465 470 475 480		
50	Leu Gly Asp Glu Ala Gln Ala Ala Ser Gly Thr Ala Arg Ala Ala Ser 485 490 495		
55	Gly Lys Ala Arg Ala Ala Ser Gly Arg Ile Arg Gln Leu Thr Leu Ala 500 505 510		
	Ala Asp Lys Gly Tyr Glu Val Val Ala Asn Leu Phe Gln Val Pro Gln 515 520 525		
	Asn Pro Val Val Asp Gly Ile Leu Ala Ser Pro Gly Val Leu Arg Gly 530 535 540		
	Ala His Asn Leu Asp Cys Val Leu Arg Glu Gly Ala Thr Leu Phe Pro 545 550 555 560		
	Val Val Ile Thr Thr Val Glu Asp Ala Met Thr Pro Lys Ala Leu Asn 565 570 575		
	Ser Lys Met Phe Ala Val Ile Glu Gly Val Arg Glu Asp Leu Gln Pro		

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	580	585	590
5	Pro Ser Gln Arg Gly Ser Phe Ile Arg Thr Leu Ser Gly His Arg Val 595	600	605
10	Tyr Gly Tyr Ala Pro Asp Gly Val Leu Pro Leu Glu Thr Gly Arg Asp 610	615	620
	Tyr Thr Val Val Pro Ile Asp Asp Val Trp Asp Asp Ser Ile Met Leu 625	630	635 640
15	Ser Lys Asp Pro Ile Pro Pro Ile Val Gly Asn Ser Gly Asn Leu Ala 645	650	655
20	Ile Ala Tyr Met Asp Val Phe Arg Pro Lys Val Pro Ile His Val Ala 660	665	670
	Met Thr Gly Ala Leu Asn Ala Cys Gly Glu Ile Glu Lys Val Ser Phe 675	680	685
25	Arg Ser Thr Lys Leu Ala Thr Ala His Arg Leu Gly Leu Arg Leu Ala 690	695	700
30	Gly Pro Gly Ala Phe Asp Val Asn Thr Gly Pro Asn Trp Ala Thr Phe 705	710	715 720
	Ile Lys Arg Phe Pro His Asn Pro Arg Asp Trp Asp Arg Leu Pro Tyr 725	730	735
35	Leu Asn Leu Pro Tyr Leu Pro Pro Asn Ala Gly Arg Gln Tyr His Leu 740	745	750
40	Ala Met Ala Ala Ser Glu Phe Lys Glu Thr Pro Glu Leu Glu Ser Ala 755	760	765
	Val Arg Ala Met Glu Ala Ala Ala Asn Val Asp Pro Leu Phe Gln Ser 770	775	780
45	Ala Leu Ser Val Phe Met Trp Leu Glu Glu Asn Gly Ile Val Thr Asp 785	790	795 800
50	Met Ala Asn Phe Ala Leu Ser Asp Pro Asn Ala His Arg Met Arg Asn 805	810	815
	Phe Leu Ala Asn Ala Pro Gln Ala Gly Ser Lys Ser Gln Arg Ala Lys 820	825	830
55	Tyr Gly Thr Ala Gly Tyr Gly Val Glu Ala Arg Gly Pro Thr Pro Glu		

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	835	840	845
5	Glu Ala Gln Arg Glu Lys Asp Thr Arg Ile Ser Lys Lys Met Glu Thr 850	855	860
10	Met Gly Ile Tyr Phe Ala Thr Pro Glu Trp Val Ala Leu Asn Gly His 865	870	875 880
	Arg Gly Pro Ser Pro Gly Gln Leu Lys Tyr Trp Gln Asn Thr Arg Glu 885	890	895
15	Ile Pro Asp Pro Asn Glu Asp Tyr Leu Asp Tyr Val His Ala Glu Lys 900	905	910
20	Ser Arg Leu Ala Ser Glu Glu Gln Ile Leu Arg Ala Ala Thr Ser Ile 915	920	925
25	Tyr Gly Ala Pro Gly Gln Ala Glu Pro Pro Gln Ala Phe Ile Asp Glu 930	935	940
	Val Ala Lys Val Tyr Glu Ile Asn His Gly Arg Gly Pro Asn Gln Glu 945	950	955 960
30	Gln Met Lys Asp Leu Leu Leu Thr Ala Met Glu Met Lys His Arg Asn 965	970	975
35	Pro Arg Arg Ala Leu Pro Lys Pro Lys Pro Lys Pro Asn Ala Pro Thr 980	985	990
	Gln Arg Pro Pro Gly Arg Leu Gly Arg Trp Ile Arg Thr Val Ser Asp 995	1000	1005
40	Glu Asp Leu Glu 1010		

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3261 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS  
(B) LOCATION:97..531

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

5  
GGATACGATC GGTCTGACCC CGGGGGAGTC ACCCGGGGAC AGGCCGTCAA GGCCTTGTTT 60  
10 CAGGATGGGA CTCCTCCTTC TACAACGCTA TCATTC GAA GTT AGT TGA GAT CTG 114  
Glu Val Ser \* Asp Leu  
1 5  
15 ACA AAC GAT CGC AGC GAT GAC AAA CCT GCA AGA TCA AAC CCA ACA GAT 162  
Thr Asn Asp Arg Ser Asp Asp Lys Pro Ala Arg Ser Asn Pro Thr Asp  
10 15 20

20

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

25 (A) LENGTH: 2827 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

35 (A) NAME/KEY: CDS  
(B) LOCATION:112..2745

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

40 GGATACGATG GGTCTGACCC TCTGGGAGTC ACGAATTAAC GTGGCTACTA GGGGCGATAC 60  
CCGCCGCTGG CTGCCACGTT AGTGGCTCCT CTTCTTGATG ATTCTGCCAC C ATG AGT 117  
Met Ser  
45 1  
GAC ATT TTC AAC AGT CCA CAG GCG CGA AGC ACG ATC TCA GCA GCG TTC 165  
Asp Ile Phe Asn Ser Pro Gln Ala Arg Ser Thr Ile Ser Ala Ala Phe  
50 5 10 15  
GGC ATA AAG CCT ACT GCT GGA CAA GAC GTG GAA GAA CTC TTG ATC CCT 213

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	Gly	Ile	Lys	Pro	Thr	Ala	Gly	Gln	Asp	Val	Glu	Glu	Leu	Leu	Ile	Pro	
	20						25				30						
5	AAA	GTT	TGG	GTG	CCA	CCT	GAG	GAT	CCG	CTT	GCC	AGC	CCT	AGT	CGA	CTG	261
	Lys	Val	Trp	Val	Pro	Pro	Glu	Asp	Pro	Leu	Ala	Ser	Pro	Ser	Arg	Leu	
	35					40				45						50	
10	GCA	AAG	TTC	CTC	AGA	GAG	AAC	GGC	TAC	AAA	GTT	TTG	CAG	CCG	CGG	TCT	309
	Ala	Lys	Phe	Leu	Arg	Glu	Asn	Gly	Tyr	Lys	Val	Leu	Gln	Pro	Arg	Ser	
					55				60						65		
15	CTG	CCC	GAG	AAT	GAG	GAG	TAT	GAG	ACC	GAC	CAA	ATA	CTC	CCA	GAC	TTA	357
	Leu	Pro	Glu	Asn	Glu	Glu	Tyr	Glu	Thr	Asp	Gln	Ile	Leu	Pro	Asp	Leu	
				70				75					80				
20	GCA	TGG	ATG	CGA	CAG	ATA	GAA	GGG	GCT	GTT	TTA	AAA	CCC	ACT	CTA	TCT	405
	Ala	Trp	Met	Arg	Gln	Ile	Glu	Gly	Ala	Val	Leu	Lys	Pro	Thr	Leu	Ser	
		85					90					95					
25	CTC	CCT	ATT	GGA	GAT	CAG	GAG	TAC	TTC	CCA	AAG	TAC	TAC	CCA	ACA	CAT	453
	Leu	Pro	Ile	Gly	Asp	Gln	Glu	Tyr	Phe	Pro	Lys	Tyr	Tyr	Pro	Thr	His	
	100					105						110					
30	CGC	CCT	AGC	AAG	GAG	AAG	CCC	AAT	GCG	TAC	CCG	CCA	GAC	ATC	GCA	CTA	501
	Arg	Pro	Ser	Lys	Glu	Lys	Pro	Asn	Ala	Tyr	Pro	Pro	Asp	Ile	Ala	Leu	
	115					120				125					130		
35	CTC	AAG	CAG	ATG	ATT	TAC	CTG	TTT	CTC	CAG	GTT	CCA	GAG	GCC	AAC	GAG	549
	Leu	Lys	Gln	Met	Ile	Tyr	Leu	Phe	Leu	Gln	Val	Pro	Glu	Ala	Asn	Glu	
				135					140					145			
40	GGC	CTA	AAG	GAT	GAA	GTA	ACC	CTC	TTG	ACC	CAA	AAC	ATA	AGG	GAC	AAG	597
	Gly	Leu	Lys	Asp	Glu	Val	Thr	Leu	Leu	Thr	Gln	Asn	Ile	Arg	Asp	Lys	
				150					155					160			
45	GCC	TAT	GGA	AGT	GGG	ACC	TAC	ATG	GGA	CAA	GCA	ACT	CGA	CTT	GTG	GCC	645
	Ala	Tyr	Gly	Ser	Gly	Thr	Tyr	Met	Gly	Gln	Ala	Thr	Arg	Leu	Val	Ala	
			165				170						175				
50	ATG	AAG	GAG	GTC	GCC	ACT	GGA	AGA	AAC	CCA	AAC	AAG	GAT	CCT	CTA	AAG	693
	Met	Lys	Glu	Val	Ala	Thr	Gly	Arg	Asn	Pro	Asn	Lys	Asp	Pro	Leu	Lys	
	180					185						190					
55	CTT	GGG	TAC	ACT	TTT	GAG	AGC	ATC	GCG	CAG	CTA	CTT	GAC	ATC	ACA	CTA	741
	Leu	Gly	Tyr	Thr	Phe	Glu	Ser	Ile	Ala	Gln	Leu	Leu	Asp	Ile	Thr	Leu	
	195					200				205					210		
55	CCG	GTA	GGC	CCA	CCC	GGT	GAG	GAT	GAC	AAG	CCC	TGG	GTG	CCA	CTC	ACA	789

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	Pro Val Gly Pro Pro Gly Glu Asp Asp Lys Pro Trp Val Pro Leu Thr	
	215 220 225	
5	AGA GTG CCG TCA CGG ATG TTG GTG CTG ACG GGA GAC GTA GAT GGC GAC Arg Val Pro Ser Arg Met Leu Val Leu Thr Gly Asp Val Asp Gly Asp	837
	230 235 240	
10	TTT GAG GTT GAA GAT TAC CTT CCC AAA ATC AAC CTC AAG TCA TCA AGT Phe Glu Val Glu Asp Tyr Leu Pro Lys Ile Asn Leu Lys Ser Ser Ser	885
	245 250 255	
15	GGA CTA CCA TAT GTA GGT CGC ACC AAA GGA GAG ACA ATT GGC GAG ATG Gly Leu Pro Tyr Val Gly Arg Thr Lys Gly Glu Thr Ile Gly Glu Met	933
	260 265 270	
20	ATA GCT ATA TCA AAC CAG TTT CTC AGA GAG CTA TCA ACA CTG TTG AAG Ile Ala Ile Ser Asn Gln Phe Leu Arg Glu Leu Ser Thr Leu Leu Lys	981
	275 280 285 290	
25	CAA GGT GCA GGG ACA AAG GGG TCA AAC AAG AAG AAG CTA CTC AGC ATG Gln Gly Ala Gly Thr Lys Gly Ser Asn Lys Lys Lys Leu Leu Ser Met	1029
	295 300 305	
30	TTA AGT GAC TAT TGG TAC TTA TCA TGC GGG CTT TTG TTT CCA AAG GCT Leu Ser Asp Tyr Trp Tyr Leu Ser Cys Gly Leu Leu Phe Pro Lys Ala	1077
	310 315 320	
35	GAA AGG TAC GAC AAA AGT ACA TGG CTC ACC AAG ACC CGG AAC ATA TGG Glu Arg Tyr Asp Lys Ser Thr Trp Leu Thr Lys Thr Arg Asn Ile Trp	1125
	325 330 335	
40	TCA GCT CCA TCC CCA ACA CAC CTC ATG ATC TCC ATG ATC ACC TGG CCC Ser Ala Pro Ser Pro Thr His Leu Met Ile Ser Met Ile Thr Trp Pro	1173
	340 345 350	
45	GTG ATG TCC AAC AGC CCA AAT AAC GTG TTG AAC ATT GAA GGG TGT CCA Val Met Ser Asn Ser Pro Asn Asn Val Leu Asn Ile Glu Gly Cys Pro	1221
	355 360 365 370	
50	TCA CTC TAC AAA TTC AAC CCG TTC AGA GGA GGG TTG AAC AGG ATC GTC Ser Leu Tyr Lys Phe Asn Pro Phe Arg Gly Gly Leu Asn Arg Ile Val	1269
	375 380 385	
55	GAG TGG ATA TTG GCC CCG GAA GAA CCC AAG GCT CTT GTA TAT GCG GAC Glu Trp Ile Leu Ala Pro Glu Glu Pro Lys Ala Leu Val Tyr Ala Asp	1317
	390 395 400	
55	AAC ATA TAC ATT GTC CAC TCA AAC ACG TGG TAC TCA ATT GAC CTA GAG	1365

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	Asn Ile Tyr Ile Val His Ser Asn Thr Trp Tyr Ser Ile Asp Leu Glu	
	405 410 415	
5	AAG GGT GAG GCA AAC TGC ACT CGC CAA CAC ATG CAA GCC GCA ATG TAC Lys Gly Glu Ala Asn Cys Thr Arg Gln His Met Gln Ala Ala Met Tyr	1413
	420 425 430	
10	TAC ATA CTC ACC AGA GGG TGG TCA GAC AAC GGC GAC CCA ATG TTC AAT Tyr Ile Leu Thr Arg Gly Trp Ser Asp Asn Gly Asp Pro Met Phe Asn	1461
	435 440 445 450	
15	CAA ACA TGG GCC ACC TTT GCC ATG AAC ATT GCC CCT GCT CTA GTG GTG Gln Thr Trp Ala Thr Phe Ala Met Asn Ile Ala Pro Ala Leu Val Val	1509
	455 460 465	
20	GAC TCA TCG TGC CTG ATA ATG AAC CTG CAA ATT AAG ACC TAT GGT CAA Asp Ser Ser Cys Leu Ile Met Asn Leu Gln Ile Lys Thr Tyr Gly Gln	1557
	470 475 480	
25	GGC AGC GGG AAT GCA GCC ACG TTC ATC AAC AAC CAC CTC TTG AGC ACG Gly Ser Gly Asn Ala Ala Thr Phe Ile Asn Asn His Leu Leu Ser Thr	1605
	485 490 495	
30	CTA GTG CTT GAC CAG TGG AAC TTG ATG AGA CAG CCC AGA CCA GAC AGC Leu Val Leu Asp Gln Trp Asn Leu Met Arg Gln Pro Arg Pro Asp Ser	1653
	500 505 510	
35	GAG GAG TTC AAA TCA ATT GAG GAC AAG CTA GGT ATC AAC TTT AAG ATT Glu Glu Phe Lys Ser Ile Glu Asp Lys Leu Gly Ile Asn Phe Lys Ile	1701
	515 520 525 530	
40	GAG AGG TCC ATT GAT GAT ATC AGG GGC AAG CTG AGA CAG CTT GTC CTC Glu Arg Ser Ile Asp Asp Ile Arg Gly Lys Leu Arg Gln Leu Val Leu	1749
	535 540 545	
45	CTT GCA CAA CCA GGG TAC CTG AGT GGG GGG GTT GAA CCA GAA CAA TCC Leu Ala Gln Pro Gly Tyr Leu Ser Gly Gly Val Glu Pro Glu Gln Ser	1797
	550 555 560	
50	AGC CCA ACT GTT GAG CTT GAC CTA CTA GGG TGG TCA GCT ACA TAC AGC Ser Pro Thr Val Glu Leu Asp Leu Leu Gly Trp Ser Ala Thr Tyr Ser	1845
	565 570 575	
55	AAA GAT CTC GGG ATC TAT GTG CCG GTG CTT GAC AAG GAA CGC CTA TTT Lys Asp Leu Gly Ile Tyr Val Pro Val Leu Asp Lys Glu Arg Leu Phe	1893
	580 585 590	
55	TGT TCT GCT GCG TAT CCC AAG GGA GTA GAG AAC AAG AGT CTC AAG TCC	1941

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	Cys	Ser	Ala	Ala	Tyr	Pro	Lys	Gly	Val	Glu	Asn	Lys	Ser	Leu	Lys	Ser	
	595					600				605					610		
5	AAA	GTC	GGG	ATC	GAG	CAG	GCA	TAC	AAG	GTA	GTC	AGG	TAT	GAG	GCG	TTG	1989
	Lys	Val	Gly	Ile	Glu	Gln	Ala	Tyr	Lys	Val	Val	Arg	Tyr	Glu	Ala	Leu	
					615					620					625		
10	AGG	TTG	GTA	GGT	GGT	TGG	AAC	TAC	CCA	CTC	CTG	AAC	AAA	GCC	TGC	AAG	2037
	Arg	Leu	Val	Gly	Gly	Trp	Asn	Tyr	Pro	Leu	Leu	Asn	Lys	Ala	Cys	Lys	
					630					635					640		
15	AAT	AAC	GCA	GGC	GCC	GCT	CGG	CGG	CAT	CTG	GAG	GCC	AAG	GGG	TTC	CCA	2085
	Asn	Asn	Ala	Gly	Ala	Ala	Arg	Arg	His	Leu	Glu	Ala	Lys	Gly	Phe	Pro	
					645				650					655			
20	CTC	GAC	GAG	TTC	CTA	GCC	GAG	TGG	TCT	GAG	CTG	TCA	GAG	TTC	GGT	GAG	2133
	Leu	Asp	Glu	Phe	Leu	Ala	Glu	Trp	Ser	Glu	Leu	Ser	Glu	Phe	Gly	Glu	
					660										670		
25	GCC	TTC	GAA	GGC	TTC	AAT	ATC	AAG	CTG	ACC	GTA	ACA	TCT	GAG	AGC	CTA	2181
	Ala	Phe	Glu	Gly	Phe	Asn	Ile	Lys	Leu	Thr	Val	Thr	Ser	Glu	Ser	Leu	
						680									690		
30	GCC	GAA	CTG	AAC	AAG	CCA	GTA	CCC	CCC	AAG	CCC	CCA	AAT	GTC	AAC	AGA	2229
	Ala	Glu	Leu	Asn	Lys	Pro	Val	Pro	Pro	Lys	Pro	Pro	Asn	Val	Asn	Arg	
						695									705		
35	CCA	GTC	AAC	ACT	GGG	GGA	CTC	AAG	GCA	GTC	AGC	AAC	GCC	CTC	AAG	ACC	2277
	Pro	Val	Asn	Thr	Gly	Gly	Leu	Lys	Ala	Val	Ser	Asn	Ala	Leu	Lys	Thr	
					710										720		
40	GGT	CGG	TAC	AGG	AAC	GAA	GCC	GGA	CTG	AGT	GGT	CTC	GTC	CTT	CTA	GCC	2325
	Gly	Arg	Tyr	Arg	Asn	Glu	Ala	Gly	Leu	Ser	Gly	Leu	Val	Leu	Leu	Ala	
					725										735		
45	ACA	GCA	AGA	AGC	CGT	CTG	CAA	GAT	GCA	GTT	AAG	GCC	AAG	GCA	GAA	GCC	2373
	Thr	Ala	Arg	Ser	Arg	Leu	Gln	Asp	Ala	Val	Lys	Ala	Lys	Ala	Glu	Ala	
					740										745		
50	GAG	AAA	CTC	CAC	AAG	TCC	AAG	CCA	GAC	GAC	CCC	GAT	GCA	GAC	TGG	TTC	2421
	Glu	Lys	Leu	His	Lys	Ser	Lys	Pro	Asp	Asp	Pro	Asp	Ala	Asp	Trp	Phe	
						755									760		
55	GAA	AGA	TCA	GAA	ACT	CTG	TCA	GAC	CTT	CTG	GAG	AAA	GCC	GAC	ATC	GCC	2469
	Glu	Arg	Ser	Glu	Thr	Leu	Ser	Asp	Leu	Leu	Glu	Lys	Ala	Asp	Ile	Ala	
						775									780		
60	AGC	AAG	GTC	GCC	CAC	TCA	GCA	CTC	GTG	GAA	ACA	AGC	GAC	GCC	CTT	GAA	2517

	Ser Lys Val Ala His Ser Ala Leu Val Glu Thr Ser Asp Ala Leu Glu	
	790 795 800	
5	GCA GTT CAG TCG ACT TCC GTG TAC ACC CCC AAG TAC CCA GAA GTC AAG Ala Val Gln Ser Thr Ser Val Tyr Thr Pro Lys Tyr Pro Glu Val Lys	2565
	805 810 815	
10	AAC CCA CAG ACC GCC TCC AAC CCC GTT GTT GGG CTC CAC CTG CCC GCC Asn Pro Gln Thr Ala Ser Asn Pro Val Val Gly Leu His Leu Pro Ala	2613
	820 825 830	
15	AAG AGA GCC ACC GGT GTC CAG GCC GCT CTT CTC GGA GCA GGA ACG AGC Lys Arg Ala Thr Gly Val Gln Ala Ala Leu Leu Gly Ala Gly Thr Ser	2661
	835 840 845 850	
20	AGA CCA ATG GGG ATG GAG GCC CCA ACA CGG TCC AAG AAC GCC GTG AAA Arg Pro Met Gly Met Glu Ala Pro Thr Arg Ser Lys Asn Ala Val Lys	2709
	855 860 865	
25	ATG GCC AAA CGG CGG CAA CGC CAA AAG GAG AGC CGC TAACAGCCAT Met Ala Lys Arg Arg Gln Arg Gln Lys Glu Ser Arg	2755
	870 875	
30	GATGGGAACC ACTCAAGAAG AGGACACTAA TCCCAGACCC CGTATCCCCG GCCTTCGCCT	2815
	GCGGGGGCCC CC	2827

### Claims

- 40 1. A birnavirus mutant which is not able to produce a native VP5 protein as a result of a mutation in the VP5 gene of the birnavirus genome, **characterised in that** the mutation comprises:
  - (i) a substitution of at least two nucleotides of the start codon of the VP5 gene, and
  - (ii) a stop codon in each of the three open reading frames in the 5'-end of the VP5 gene.
- 45 2. A birnavirus mutant according to claim 2, **characterised in that** the birnavirus is infectious bursal disease virus (IBDV).
- 50 3. A birnavirus mutant according to claims 1-2, **characterised in that** the mutation is in the genome of a virulent field virus.
4. A birnavirus mutant according to claim 2, **characterised in that** the mutation is in the genome of a vaccine strain, preferably in vaccine strain D78.
- 55 5. A birnavirus mutant according to claims 2-4, **characterised in that** the mutant has a mutated start codon and three stop codons in the 5'-end of the VP5 gene as shown in SEQ ID No: 7.
6. A birnavirus mutant according to claims 2-5, **characterised in that** the IBDV expresses a chimeric VP2 protein

comprising virus neutralising epitopes of different antigenic IBDV types.

7. A vaccine against a birnavirus infection in animals, **characterised in that** it comprises a birnavirus mutant according to claims 1-6 and a pharmaceutically acceptable carrier.
8. A method for the attenuation of virulence of a birnavirus in an animal, comprising the step of introducing a mutation in the VP5 gene as a result of which the birnavirus is not able to produce a VP5 protein.
9. A method according to claim 9, wherein the mutation is a substitution.
10. A method according to claims 8-9, wherein the birnavirus is infectious bursal disease virus (IBDV).
11. A method according to claims 8-10, wherein the mutation is in the genome of a virulent field virus.
12. A method according to claims 8-11, wherein the mutation comprises a substitution of at least two nucleotides of the start codon of the VP5 gene.
13. A method according to claim 12 wherein the mutation comprises additionally one or more stop codons in the 5'-end of the VP5 gene.
14. A method according to claim 13, wherein the mutation comprises a stop codon in each of the three open reading frames.
15. A method according to claim 14, wherein the mutation is in the start codon and comprises three stop codons in the 5'-end of the VP5 gene as shown in SEQ ID No: 7.

#### Patentansprüche

1. Eine Birnavirus Mutante, die aufgrund einer Mutation im VP5 Gen des Birnavirus Genoms kein natives VP5 Protein produzieren kann, **dadurch gekennzeichnet, dass** die Mutation:
  - (i) eine Substitution von mindestens zwei Nukleotiden des Startcodons des VP5 Gens, und
  - (ii) ein Stopcodon in jedem der drei offenen Leserahmen am 5'-Ende des VP5 Gens.
 umfasst.
2. Eine Birnavirus Mutante gemäß Anspruch 1, **dadurch gekennzeichnet dass** das Birnavirus ein Infektiöses Bursitis Virus (IBDV) darstellt.
3. Eine Birnavirus Mutante gemäß Ansprüchen 1-2, **dadurch gekennzeichnet, dass** die Mutation im Genom eines virulenten Feldvirus ist.
4. Eine Birnavirus Mutante gemäß Anspruch 2, **dadurch gekennzeichnet, dass** die Mutation im Genom eines Impfstoff-Stammes, vorzugsweise im Impfstoff-Stamm D78, ist.
5. Eine Birnavirus Mutante gemäß Ansprüchen 2-4, **dadurch gekennzeichnet, dass** die Mutante ein mutiertes Startcodon und drei Stopcodons am 5'-Ende des VP5-Gens wie in SEQ ID No:7 dargestellt besitzt.
6. Eine Birnavirus Mutante gemäß Ansprüchen 2-5, **dadurch gekennzeichnet, dass** das IBDV ein chimäres VP2 Protein exprimiert, das Virus-neutralisierende Epitope von unterschiedlichen, antigenen IBDV-Typen umfasst.
7. Ein Impfstoff gegen eine Birnavirus Infektion in Tieren, **dadurch gekennzeichnet, dass** er eine Birnavirus Mutante gemäß Ansprüchen 1-6 und einen pharmazeutisch verträglichen Träger umfasst.
8. Verfahren zur Abschwächung der Virulenz eines Birnavirus in einem Tier, welches den Schritt des Einführens einer Mutation in das VP5 Gen umfasst, als Folge dessen das Birnavirus kein VP5 Protein produzieren kann.

9. Verfahren gemäss Anspruch 8, worin die Mutation eine Substitution darstellt.
10. Verfahren gemäss Ansprüchen 8-9, worin das Birnavirus ein Infektiöses Bursitis Virus (IBDV) darstellt.
11. Verfahren gemäss Ansprüchen 8-10, worin die Mutation im Genom eines virulenten Feldvirus ist.
12. Verfahren gemäss Ansprüchen 8-11, worin die Mutation eine Substitution von mindestens zwei Nukleotiden des Startcodons des VP5 Gens umfasst.
13. Verfahren gemäss Anspruch 12, worin die Mutation zusätzlich ein oder mehrere Stopcodons am 5'-Ende des VP5-Gens umfasst.
14. Verfahren gemäss Anspruch 13, worin die Mutation in jedem der drei offenen Leserahmen ein Stopcodon umfasst.
15. Verfahren gemäss Anspruch 14, worin die Mutation im Startcodon ist und drei Stopcodons am 5'-Ende des VP5-Gens wie in SEQ ID No:7 dargestellt umfasst.

## Revendications

1. Un mutant du birnavirus qui est incapable de produire une protéine VP5 native, résultant d'une mutation dans le gène VP5 du génome du birnavirus, **caractérisé en ce que** la mutation comprend :
  - i) une substitution d'au moins deux nucléotides du codon de départ du gène VP5, et
  - ii) un codon d'arrêt dans chacun des trois cadres ouverts de lecture dans l'extrémité 5' du gène VP5.
2. Un mutant du birnavirus selon la revendication 1, **caractérisé en ce que** le birnavirus est le virus de la bursite infectieuse aviaire (IBDV : *Infections Bursal Disease Virus*).
3. Un mutant du birnavirus selon la revendication 1 ou 2, **caractérisé en ce que** la mutation est dans le génome d'un virus sauvage virulent.
4. Un mutant du birnavirus selon la revendication 2, **caractérisé en ce que** la mutation est dans le génome d'une souche de vaccin, de préférence dans la souche de vaccin D78.
5. Un mutant du birnavirus selon l'une des revendications 2 à 4, **caractérisé en ce que** le mutant a un codon de départ ayant subi une mutation et les trois codons d'arrêt dans l'extrémité 5' du gène VP5 tel que représenté dans la SEQ ID n° 7.
6. Un mutant du birnavirus selon l'une des revendications 2 à 5, **caractérisé en ce que** l'IBDV exprime une protéine chimère VP2 comprenant des épitopes neutralisant des virus de différents types antigènes d'IBDV.
7. Un vaccin contre une infection par le birnavirus chez des animaux, **caractérisé en ce qu'il** comprend un mutant du birnavirus selon l'une des revendications 1 à 6 et un support pharmaceutiquement acceptable.
8. Un procédé pour atténuer la virulence d'un birnavirus chez un animal, comprenant l'étape consistant à introduire une mutation dans le gène VP5 pour que le birnavirus soit incapable de produire une protéine VP5.
9. Un procédé selon la revendication 9, dans lequel la mutation est une substitution.
10. Un procédé selon la revendication 8 ou 9, dans lequel le birnavirus est le virus de la bursite infectieuse aviaire (IBDV).
11. Un procédé selon l'une des revendications 8 à 10, dans lequel la mutation est dans le génome d'un virus sauvage virulent.
12. Un procédé selon l'une des revendications 8 à 11, dans lequel la mutation comprend une substitution d'au moins deux nucléotides du codon de départ du gène VP5.

13. Un procédé selon la revendication 12, dans lequel la mutation comprend en outre un ou plusieurs codons d'arrêt dans l'extrémité 5' du gène VP5.
14. Un procédé selon la revendication 13, dans lequel la mutation comprend un codon d'arrêt dans chacun des trois cadres ouverts de lecture.
15. Un procédé selon la revendication 14, dans lequel la mutation est située dans le codon de départ et comprend trois codons d'arrêt dans l'extrémité 5' du gène VP5 tel que représenté dans la SEQ ID n° 7.

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Figure 2

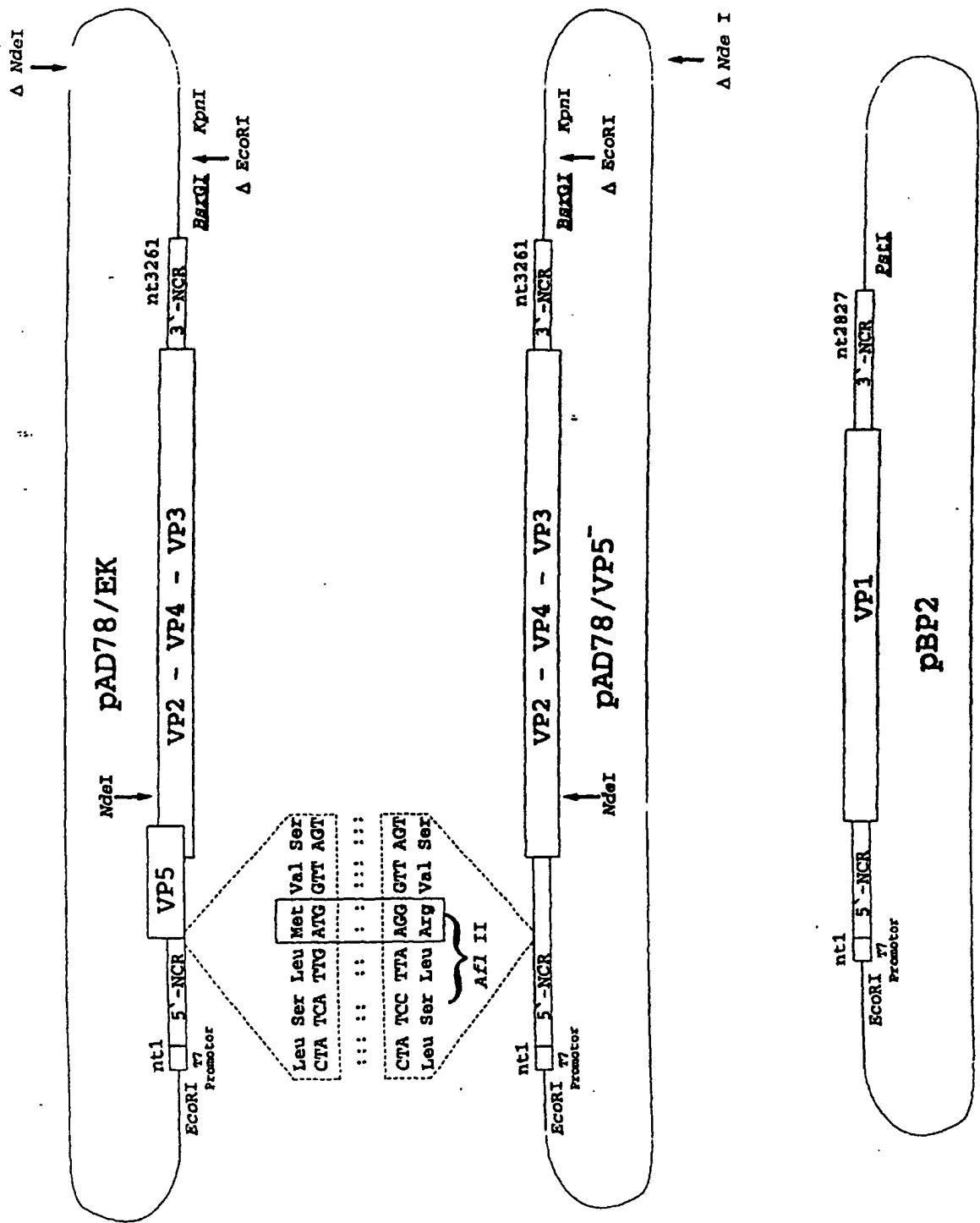
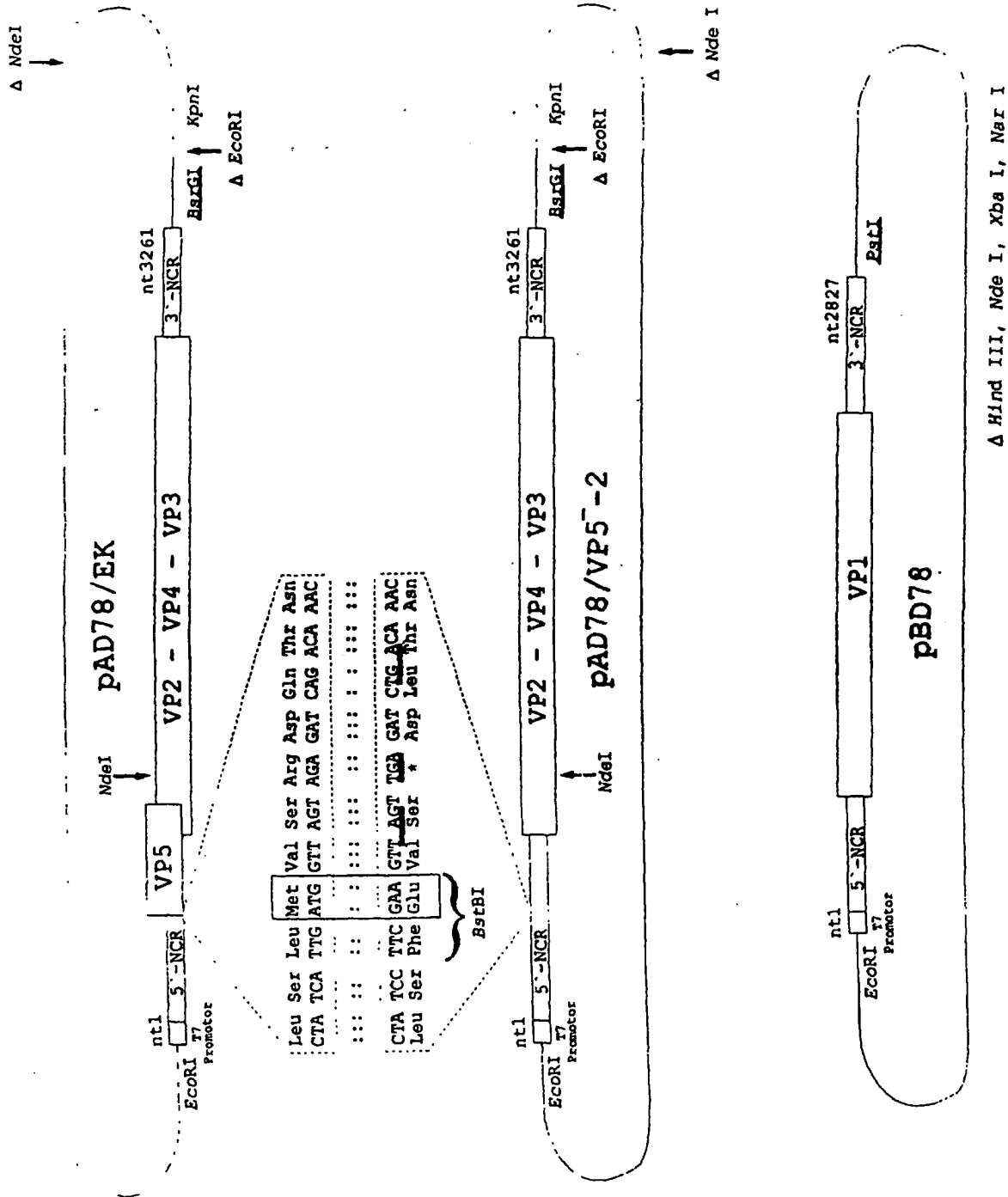


Figure 3



Δ Hind III, Nde I, Xba I, Nar I

Figure 4

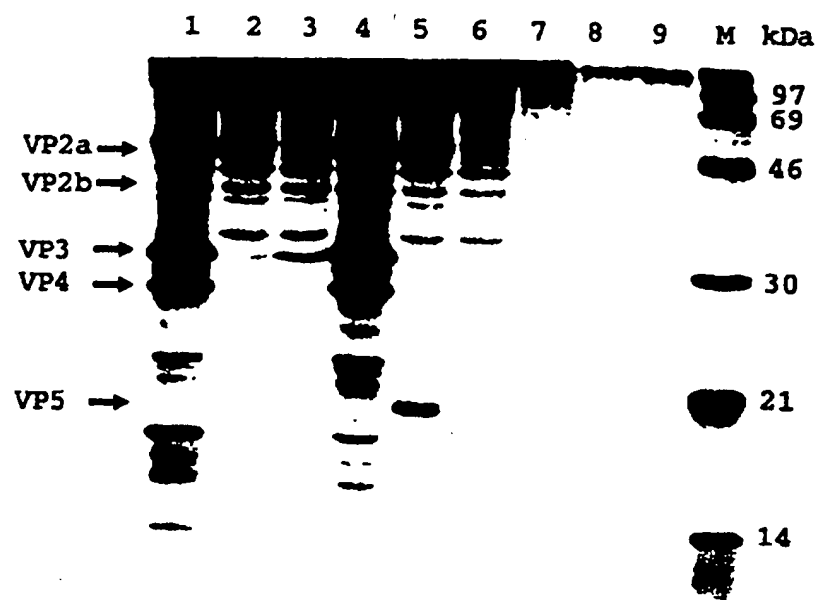
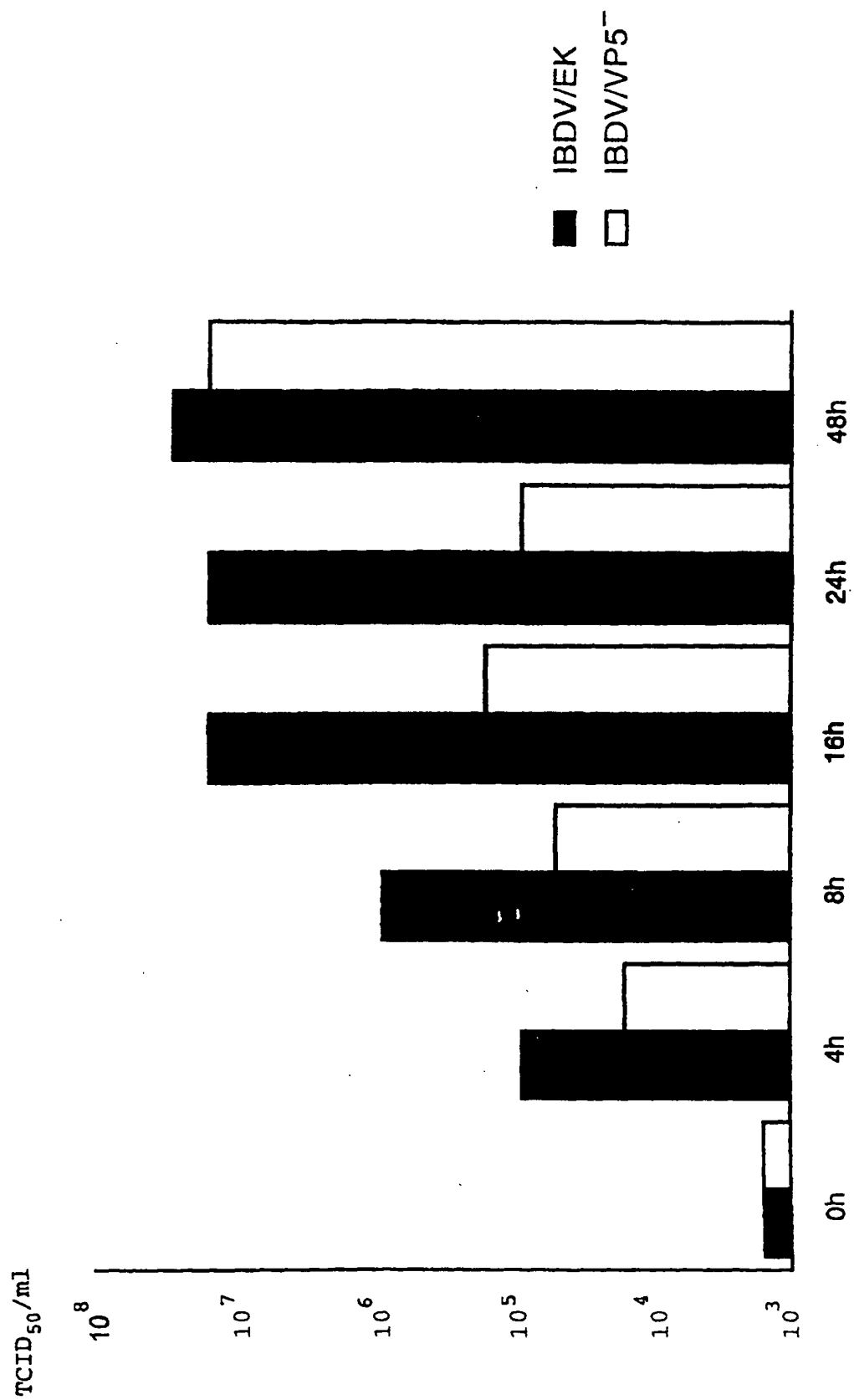


Figure 5



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